BLOOD COAGULATION, HEMORRHAGE AND THROMBOSIS

Methods of Study

edited by

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AND

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GRUNE & STRATTON • NEW YORK • LONDON

ACKNOWLEDGMENTS

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This book was published originally as *The Coagulation of Blood Methods of Study*, © 1955 GRUNE & STRATTON, INC.

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PREFACE TO SECOND EDITION

The play's the thing wherein I'll catch the conscience of the king.
Hamlet: Act II, Sc. 2
William Shakespeare, 1604

This book is dedicated to the memory of its author, the late Leandro M Tocantins, M.D., whose untimely death interrupted its completion. It expresses one of his specific interests in hematology, his great devotion to blood coagulation and his faith in hemostasis as fruitful fields of exploration from which ultimately the patient must derive the real benefits of clinical science. Such philosophy is only implicitly evident in the nature of the book, for it presents only a compilation of tests and preparatory methods in the field of blood coagulation, hemorrhage and thrombosis. Those who helped to plan the first and second editions, who collaborated in publication and who knew the author are quite cognizant of his philosophy. His devotion to these concepts and his appreciation of the importance of methodology were powerful motivating forces behind this work. His fond and cherished hope was always that the methodology in this book would not only be useful to the expert but would serve also to stimulate the less experienced but equally interested investigator faced with the problem of selecting an assay or preparative procedure for research in the field of blood coagulation and hemostasis.

The quotations reproduced here and elsewhere in the book were his own selections The main quotation was paraphrased from Act I, Scene 5 of Shakespeare's Hamlet "There are more things in heaven and earth, Horatio, than are dreamt of in your philosophy" Scribbled on scraps of paper and labeled "For Preface," they, as well as other memorabilia, indicate that not only were his thoughts frequently "with the book" but that he was keenly interested in and aware of the importance of hemostasis, at the same time fully realizing the potentialities as well as the limitations of clotting phenomena Hemostasis is certainly the "king" whose essence is reflected by the experimental and clinical manifestations of blood coagulation. He recognized that hemorrhage and thrombosis were among the commonest manifestations of disease, that hemorrhage can be clinically recognized and approximately estimated but that thrombosis was difficult to recognize and estimate He believed also that our knowledge of blood coagulation rendered somewhat easier the investigation of hemorrhage but

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greatly appreciated is clearly evident from the files of the late senior editor. My personal indebtedness to the Contributors is equally great, especially for their cooperation, advice, and patience.

Acknowledgments and thanks are due to many individuals, some perhaps unknown to me. The members of an earlier Subcommittee on Thrombosis and Hemorrhage of the National Academy of Sciences, National Research Council, under the chairmanship of Dr. Kenneth M. Brinkhous participated in the launching of the second edition. More recently, Dr. Edwin Coyl of the Division of Medical Sciences, National Academy of Sciences, and the members of the present Subcommittee under the chairmanship of Dr. C. Lockard Conley, were extremely helpful in the transition stage of editors. The assistance, the interest and the encouragement of the members of the Subcommittees are greatly appreciated

Special acknowledgments and thanks are due to Drs. K. M. Brinkhous, C. Lockard Conley, Louis B Jaques and Walter H Seegers for frequent advice about manuscripts and other matters; to Dr Allan J Erslev, Director of the Cardeza Foundation for encouraging the completion of the book and for making available various facilities of the Cardeza Foundation at Jefferson Medical College; to Dr. George G. Grannis, Dr. Ruth R Holburn, Miss Margaret DeSipin and Miss Muriel Means for proofreading the galley, to Mrs. Barbara Segel, Mrs. Bella Sigal and Mrs Eileen O'Leary for secretarial help and to Miss Isabel Considine for library assistance. The publisher deserves special thanks for his continued interest and patience under these unusual circumstances and for much valuable advice in the final stages of the preparation of this book

LOUIS A. KAZAL
Philadelphia, Pennsylvania
1964

that the local nature of thrombosis in terms of changes of the vessel wall, seldom influenced by systemic changes, complicated the examination and control of thromboembolic disease. The opportunity to record these thoughts in his own words was never realized; however, the inclusion in the second edition of Sections on Hemorrhage and Thrombosis reflects *this* great interest in hemostasis and remains as testimony of his forward thinking. These sections extend manyfold the usefulness of the book to the clinician as well as to the fundamental researcher.

The second edition, like the first, was designed to bring to the experienced investigator, as well as to the beginner with an interest in blood coagulation, a group of methods for quick and authoritative reference and with unusual procedural detail. Although this edition has been greatly enlarged, complete coverage, a goal which is obviously difficult to attain in this modern day of rapidly expanding technology, is not fully achieved, nor was it intended. Nevertheless, an extremely useful cross-section of available procedures, dealing with the measurement or preparation of coagulant, anticoagulant, fibrinolytic and hemostatic factors or activities by experts in the field, has been gathered under one cover and should prove of value to the student of coagulation and hemostasis. As with the first edition, methodology rather than theory has been emphasized, and experimental detail was given priority over discussion.

As friend and colleague, the associate editor is grateful for the opportunity to complete the unfinished work. For the most part, the scope and general plan of the book, the selection of its methods and their contributors, and the "harvesting" of the contributions had already been accomplished Nevertheless, much remained to be done in the form of editing, organizing contents, filling in some obvious gaps in subject matter and verifying many aspects of the work In all of this the associate editor has endeavored to carry out fully the intent of the original designer, frequently in the light of apparent contradictions arising from ignorance of earlier unknown arrangements between editor and contributor Although there was some familiarity with the project, the work had to be guided and problems unraveled by searching files of correspondence and notes accumulated over several years. Except for a partial outline, in which many contributions did not appear, and the manuscripts, the only recorded evidence of final plans and intentions were a few paragraphs of an unfinished introduction and the two quotations from Hamlet The numerous notations and references found in these files, however, attest to the conscientious thinking that had gone into the planning of this book

Credit for any success enjoyed by this book goes without question to its Contributors, without whom such a critically analyzed compilation of methods would be difficult to achieve That their contributions were

INTRODUCTION

Hemorrhage and thrombosis represent but two pathologic manifestations of an essential physiologic process, hemostasis. The process appears to be largely dependent upon two interrelated mechanisms One is blood coagulation, or fibrin clotting, with its complex biochemical reactions, largely enzymatic in nature. The other, possibly even more complex, is platelet agglutination, or cellular clotting, resulting from a series of events characterized by morphologic degradation and associated biochemical changes. Initially, the hemostatic plug consists mainly of the platelet component. Intimately related to these fundamental hemostatic mechanisms is still another basic physiologic process, fibrinolysis. In the last three decades there has been a tremendous growth in our knowledge of the biochemistry, physiology and pathophysiology of the blood clotting mechanism This growth reached a peak in the early 1950's with the recognition of many new blood clotting factors or activities and many different new hemorrhagic states Only in the last few years has there been a renaissance in the fields of cellular clotting and fibrinolysis comparable to that seen earlier in blood coagulation. In all of these areas, the vehicle that made possible the many important discoveries of the recent past was improved methodology Most important have been tests and bioassays in which the end point is the formation of a fibrin clot or a platelet aggregate, or the proteolysis of a preformed clot Less often, there have been certain in vivo standardized occurrences, as production or prevention of a hemorrhagic state, or the experimental production of a thrombus or thrombolysis These methods have been designed in many laboratories and were usually reported only incidentally in research papers in a variety of journals Today these procedures frequently furnish the investigator in hemostasis with his basic tools

The first edition of this book was based on a simple concept the young investigator in blood coagulation should have available for his use a manual of the most important and more commonly used methods, described either by the authors of the methods or by investigators who have had long experience with a given procedure There should be no favored procedures, and all should be thoroughly described, with the helpful hints that usually remain as the lore of that laboratory in which the method was developed The concept was that of Dr Leandro Tocantins, and was presented by him to the Panel on Blood Coagulation of the National Research Council in the early 1950's The panel, then under the chairmanship of Dr Walter H Seegers, endorsed the proposal after some discussion and the task of assembling and editing the volume fell to Dr Tocantins The usefulness of

PREFACE TO FIRST EDITION

At almost every meeting of the Panel on Blood Coagulation of the National Academy of Sciences, National Research Council the members are called upon to decide on the merits of proposals for research in this field The impression has often been left in the minds of the members that many such proposals have come from persons who, though actuated by a sincere desire to investigate some phase of the subject of clotting, were often unacquainted with the methods involved. It seemed to the members of the panel that a few such workers might be helped if a compendium of these methods were prepared to which investigators could refer for general guidance. From the first, it was pointed out that this should be no attempt to set down such methods rigidly as "standard." Even if this were possible, it would, of course, not be desirable. Since the principle functions of the Panel are to judge, encourage and facilitate research, the concept of setting definitive standards becomes naturally abhorrent.

It was with these thoughts in mind that the Panel on Blood Coagulation commissioned me to prepare such a compendium. A general outline of the topics was drawn up and submitted to the Panel members and other interested workers, each of whom assumed the responsibility of writing the topics assigned to him. The project was promptly endorsed by the Chairman of the Division of Medical Sciences of the National Academy of Sciences, National Research Council, Dr Milton C. Winternitz, and subsequently by his successor, Dr R. Keith Cannan The members of the Panel who participated in the deliberations and in the preparation of the compendium were: Benjamin Alexander, J Garrott Allen, Kenneth M. Brinkhous, Louis B Jaques, Robert L MacMillan, J Haskell Milstone, W H Seegers, L M Tocantins

From the first, Dr Seegers, the Chairman of the Panel, supported the effort enthusiastically and contributed much helpful advice and guidance.

The decision to include any method rested principally on the familiarity of the contributors with the technic and their estimate of its importance It is fully realized that other technics exist, unquestionably as important and useful as those described, but limitations of space have made it impossible to include all but a few of those originally selected Dr Margaret Sloan, Executive Assistant to the Chairman of the Division of Medical Sciences, displayed a continued interest in furthering the work The Publishers, with their already established reputation of encouraging medical research, have been of much assistance to the Committee in fulfilling our objectives.

Leandro M Tocantins
Philadelphia, Pennsylvania
December, 1954

"We have been looking too long at the problem of hemostasis through the keyhole of blood coagulation. Paraphrasing Hamlet: 'There are more things in Hemostasis than are dreamt of in our philosophy of Blood Coagulation.'"

L. M. Tocantins, M.D.
1963

the volume has been obvious to all workers in the field. The large number of recurring references to methods which were best described in this book have saved untold numbers of pages of journals. The exhaustion of the printing of the first volume occurred several years ago. Many readers may have had my own experience—this book never seemed to be on the shelf when wanted, as it had been borrowed by some student or associate in connection with his experiments.

The conception of the second edition was much the same as for the first edition. Those who knew Dr. Tocantins and his writings were well aware that his main interest was in hemostasis and its multitudinous aberrations With the exhaustion of the first edition and the extension of knowledge into new areas, he proposed an expanded second edition to the same group at the National Research Council, now known in keeping with the times as the Subcommittee on Hemorrhage and Thrombosis. The "methods book," as it was commonly called, had proven itself and the proposal was immediately approved Enlargement of the contents to include new procedures in blood coagulation and new sections on thrombosis and fibrinolysis was planned in detail, and the assembly of the various chapters was nearly complete at the time of the editor's untimely death It is unfortunate that he did not live to see the conclusion of this endeavor so dear to his heart. He would have reason to be proud of the masterful way in which his associate, Dr Louis A. Kazal, has carried out the over-all plan and brought it to full fruition This second edition is a tribute to the foresight and effectiveness of both editors in bringing the present volume to all workers in the field Particular thanks are due to Dr. Kazal, for without his devotion and patience, this work could not have been completed

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National Research Council
August 17, 1964
Chapel Hill, North Carolina

In Memoriam

LEANDRO M. TOCANTINS, M.D.

1901-1963

On March 22, 1963, hematology and medicine suffered a great loss in the untimely death of one of its most loyal and distinguished members, Leandro M. Tocantins, M D., The Thomas Drake Martinez Cardeza Professor of Clinical Medicine and Hematology, and the Director of the Charlotte Drake Cardeza Foundation at Jefferson Medical College. The imminence of his passing was not apparent to his associates who saw him daily, for in his own quiet and unassuming manner, personal problems and difficulties were seldom mentioned and never discussed at length. The illness of which he obviously was aware was no exception. Thus, the broad and intensive life of scientific and educational pursuits continued, quietly, deliberately and at a remarkable pace, to be suddenly terminated by coronary occlusion late that day at a program committee meeting of an institution he always had held in highest esteem—the historic College of Physicians of Philadelphia. Dr. Tocantins' passing was equally a great loss to Jefferson Medical College and Hospital, which he served faithfully and in so distinguished a manner for thirty-three years, and to the Charlotte Drake Cardeza Foundation, for whose present scientific development, expansion and success he was largely responsible.

The personal loss of the teacher, researcher, clinician, physician or just friend has saddened many hearts here and abroad. Those who had the privilege of knowing him will remember unusual and admirable qualities that distinguished his person his quiet, humble, gentle and unassuming manner that concealed an intense energy and drive for work, a deep thinking and incisive mind, coupled with a great curiosity and a keen power of observation for scientific facts, a love for the science as well as the practical art of medicine with an unusual ability to blend these for the good of the patient; a humanitarian approach to the patient, an interest and great confidence in the neophyte investigator, whom he constantly sought and nurtured with the hope that some would find their life work in the study of blood coagulation or some aspect of hematology, an interest in the furtherance of medical education of the fellow, the resident and the medical student These reflections document only some of the unusual aspects of a life otherwise rich in scientific accomplishment

A hard and indefatigable worker, a greater part of his daily effort was given unselfishly to the interest of others to colleagues in science, to patients, to administration problems, to teaching, to consultations and to



Leandro M. Tocantins, M.D.

(This portrait was painted by S George Phillips and will hang in the Tocantins Memorial Library, in the Cardeza Building of Jefferson Medical Center, Philadelphia, Pennsylvania.)

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A hard and indefatigable worker, a greater part of his daily effort was given unselfishly to the interest of others to colleagues in science, to patients, to administration problems, to teaching, to consultations and to

the many "unclassified" demands of a busy physician's day. His earlier years were enriched by a greater opportunity for investigation at the bench, and clinical research at the bedside, but in later years the multiplication of responsibilities made it more and more difficult to engage personally in experimental work. This did not lessen the intense desire for knowledge and truth. His goals and objectives were pursued through the men and women whom he stimulated with his teaching and guidance Many investigators and physicians sought his assistance for their problems. For his colleagues at Jefferson and his staff at the Cardeza Foundation he provided the kind of stimulation and the atmosphere of independent thought which molded interest and contributed to progress in investigation, and he provided the physical facilities for pursuit of these ideas in research

In spite of all obligations, however, the patient remained always and to the end, the point of closest contact with clinical and physiological science, which he dearly loved. His ultimate goal in life was the welfare of the patient, and he expressed this so well in his talk, "To Serve the Patient," which he presented in 1961 to the incoming Freshman class of Jefferson Medical College [1] A quotation from this is apropos "You cannot begin to serve your patient until you know him, and you cannot begin to know him until you serve him" This dedication to the patient was clearly evident to fellows and associates with whom he made daily rounds in the clinic It embodies the human quality and spirit that only those closely working with him were privileged to experience.

Dr Tocantins' world-wide reputation in hematology, and especially in the field of blood coagulation and hemostasis, was founded on a long and arduous series of fundamental and clinical investigations on the properties and function of blood platelets, the action of antiplatelet antibodies, prothrombin, antithromboplastin and anticephalin, the coagulability and fluidity of blood, the lipid coagulants and anticoagulants, the clinical management of hemorrhagic states and especially his investigations of hemophilia in all of its aspects both from the practical viewpoint of therapy and the theoretical viewpoint of the role of inhibitors The controversial inhibitor theory, promoted for so long a time alone, has stimulated a trend of thought which may open up new paths to further exploration of blood coagulation phenomena

His interest in other areas of hematology were equally important The preservation and transplantation of bone marrow received much attention in recent years Erythrokinetic studies, the hemoglobinopathies, leukemia and anemia were favorite subjects of research The startling beneficial effect of hypnosis as an adjunct to the control of bleeding which plagues the extraction of teeth in hemophiliacs, and the potential beneficial effect of epsilon-aminocaproic acid for controlling fibrinolytically induced bleeding

in prostatectomy were for him a never-ending source of interest. The exploration of the action of this amino acid for correcting the prolonged coagulation time of hemophilic blood was one among many other fascinating projects, whose fuller development he did not live to experience, but of which he saw sufficient to comment frequently about the remarkable and unexpected observations that can be made in the study of blood coagulation and hemostasis.

More than two hundred publications since 1930 attest to his practice of reading and disseminating the product of his labor by writing These include the present volume as well as the renowned *Progress in Hematology* series His pet project in later years was the establishment of a library devoted primarily to the collection of books, periodicals, reprints and items of information about blood and blood diseases and related scientific subjects.

His extensive investigations brought him honors, consultantships and appointments to committees and panels The recently dedicated Tocantins Memorial Library of the Cardeza Foundation is a fitting tribute to his lifelong devotion to hematology. On October 12, 1960, the University of Brazil conferred upon him an honorary degree at Rio de Janeiro for his interest in medical education and research Maupin very aptly remarked [2] "Avec L. M Tocantins disparâit un des pionniers de l'hématologie mais aussi de l'hémostase. Ne peut-il pas être considéré comme l'un des ≪pères≫ des plaquettes?" and perhaps Dr Tocantins will best be remembered for his extensive work with platelets His numerous positions and appointments need not be mentioned here in detail for they have been chronicled elsewhere [1,4,5,6,7] Besides the directorship of the Cardeza Foundation 1954) and the Cardeza Professorship (1959), he was Consulting Physician to the Pennsylvania Hospital and Head of its Department of Hematology, and Consultant for the U S Naval Hospital in Philadelphia His editorial work for the Journal of Blood, and the American Journal of Physiology was undertaken in the spirit of service to others As a member of the National Research Council Subcommittee on Thrombosis and Hemorrhage (1952), and of the Hematology Study Section of the National Institutes of Health (1956), he gave unstintingly of time and interest to its work, which he considered to be of immense importance to the advancement of blood coagulation and hemostasis The Cardeza Foundation with its splendid laboratories, blood donor facilities and Library will remain as a living token of his devotion to the patient, the scientist and the institution, all of which he served so well

LOUIS A. KAZAL
Philadelphia, Pennsylvania
1964

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SECTION A

BLOOD COAGULATION

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[1] L. M. Tocantins: J A.M.A. *178* 568, 1961.
[2] B. Maupin: Hemostase *3*(3), vii, 1963
[3] Jefferson Med. College Alumni Bulletin: *11:* (6) 12, 1960; *12*· (4), 14, 1961; *1*
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SECTION A

BLOOD COAGULATION

CHAPTER I

PROCESSING OF BLOOD, PREPARATION OF GLASSWARE AND REAGENTS

1. The Collection of Blood for Studies on Coagulation

L. M. TOCANTINS

It is not generally realized by most students of blood coagulation and those charged with the responsibility of clinical measurements of the rate of blood clotting, that the mode of collection of the blood sample affects the results of this work considerably It may be worthwhile therefore, to state in detail what we consider to be minimum standards for the conditions surrounding the collection and storage of blood from the vessels of man and experimental animals These remarks apply solely to the collection of blood for studies on blood coagulation and its various factors and not necessarily to the collection of blood for biochemical or cytological studies The fact that a defective collection technique will often be satisfactory for the latter studies, has often misled many research and clinical workers into the belief that the same applies to studies on blood coagulation

1 Site for Collection of Blood A readily accessible vein of good caliber such as that of the forearm in men or of the leg or arm vein in dogs is adequate The hair over the area should be clipped if it interferes with visualization and approach to the vein, but the skin itself should not be injured during the clipping or shaving The skin overlying the vein should be cleaned with water and alcohol In order to be sure of a clean rapid venepuncture, the vein should be distended by applying a tourniquet proximal to the site of the puncture The tourniquet should be adjusted critically so that the arterial flow is adequate but the venous return is cut off Between one and two minutes should elapse before the vein is punctured to insure an adequate supply of blood and a taut distended readily punctured vein The blood should be aspirated into the syringe or allowed to flow out

of the vein into a vessel at the rate of no less than 0.5 ml. per second. The rate of aspiration of the blood should be adjusted to the rate of flow so that the negative pressure applied by the piston is not in great excess of that needed to aspirate the blood, for, in such case, air bubbles will enter the syringe between the piston and barrel or between the tip of the syringe and the hub of the needle.

2. Needles The needle should be sharp with a bevel no longer than 3 mm and the gauge should be preferably 18 or at the minimum, 20. Whenever possible it is preferable to work with the greater gauges (17 or 16). No needle smaller than 20 should be used. Disposable needles have been found to be excellent.

3 Syringes Tight fitting syringes, coated on the internal surface with silicone or mineral oil should be used. If an anticoagulant is to be added to the blood, the correct amount of the chemical in as small a volume as possible should be placed in the syringe, thus allowing the blood to come into contact with the anticoagulant immediately after it leaves the vessel. It is desirable that at least two syringes be employed, the first for the collection of 1 or 2 ml of blood to be discarded and replaced by a second syringe containing the anticoagulant After the required amount of blood has been aspirated, the syringe should be removed and tilted four or five times to insure mixture of the anticoagulant and blood

4 Silicone coated glassware or polystyrene (plastic) surfaces should be used for the centrifugation and storing of blood and plasma. The syringe is emptied of blood by placing the tip of the syringe against the side of the siliconized tube and allowing the blood to run down (and not be squirted) *on the side of the tube, without frothing or excessive turbulence. The maximum* g and time employed for centrifugation should be stated The temperature in the centrifuge should not rise above 30°C during centrifugation, and it is best kept at between 5 and 10°C

If plasma is to be separated, this should be done immediately after collection of the blood If platelet-poor plasma is desired, only the upper three-fourths of the plasma layer, after centrifugation, should be aspirated Siliconized glassware should be used in pipetting the plasma from one centrifuge tube to another, and no reflux of plasma from the pipette back to the tube should be allowed

A specimen of blood unsuitable for clotting studies may result if any or all of the following occur

1 If there is difficulty in entering the vein and more than one trial is made at puncturing it If such is the case, the fact should be noted by the operator in reporting the results

2 If the supply of blood in the veins is so restricted that a rapid aspiration is impossible This may be due to malposition or obstruction of

the needle, or inadequate filling of the vein with blood because of defective application of the tourniquet.

3. Entrance of air bubbles into the syringe while the blood is being withdrawn One or two air bubbles are permissible since these may come from the tip of the syringe or the needle itself. Repeated entrance of bubbles into the syringe during aspiration is objectionable, since it interferes with the blood-silicone interface by enlarging the air-blood interface.

4. Slow aspiration of blood into the syringe lengthens the time that the blood is out of the vessels without being adequately mixed with the anticoagulant The use of large volume syringes (30 ml or more) is undesirable since it lengthens the time required for aspirating enough blood to fill the syringe.

2. Selection of Surfaces for Needles, Syringes, Collection Sets, Containers and Test Tubes to Be Used in Clotting Studies

L. B. JAQUES

Many surfaces have been and are used for this purpose Selection is usually made (consciously or unconsciously) on the basis of availability of material, availability of suitable form, cost, machinability, transparency, *ease of cleaning, restoration of surface after use, effect on clotting* These properties are summarized in table 1· + indicates favourable, − indicates unfavourable property of particular surface ± property is favourable or unfavourable depending on use. Cleaning is discussed separately. With all surfaces, a very great problem (sometimes insurmountable) is removing the cleaning agent used

Details for application[6] of a series of coatings which can be sterilized by autoclaving and are of sufficient variety to cover all laboratory needs are given below

Application of Silicone (for glassware) Liquid application is described in section 4. The vapor treating material (Dri-Film 9977) lends itself readily to the siliconization of large amounts of equipment in a minimum of time All glassware must be properly washed with soap and water 24 hours before application of the silicone. After washing, the glassware is thoroughly rinsed in warm tap water followed by hot pyrogen-free distilled

water. It is then placed in a hot air oven and dried for 2 hours at 120°C. Following this, it is allowed to stand overnight at room temperature in order to equilibrate with the moisture in the atmosphere. This overnight equilibration is absolutely essential as the vapor will not react with or adhere to a completely dry surface. The glassware is put in cardboard cartons, or other similar containers and placed under a chemical hood. About 30 cc of Dri-Film (No. 9977) is placed in a gas washing bottle and air is bubbled slowly through it. The resultant vapor is directed through a rubber tube and glass nozzle into each of the cartons containing glassware. The tube should only be held in each carton long enough for the operator to count slowly to six. The gas bottle is then disconnected and a stream of filtered compressed air is directed into each carton for the same period of time in order to displace the extra methyl chlorosilane vapor. The glassware is then removed from the cartons, rinsed three times with hot pyrogen-free distilled water, to free it of the hydrochloric acid which has been generated. It is now ready for sterilization or immediate use as desired.

Application of Arquad (for needles) Needles after cleaning are boiled in a 1 per cent solution of Arquad for two minutes, then rinsed and allowed to dry

Application of Lacquer Silicone (for accessible metal surfaces). The materials to be treated are prepared by dipping or brushing with the resin solution and are allowed to drain. They should be placed in a vertical position to allow complete drainage. They are left in this position for 30 minutes, allowing the resin to air dry so that it will not blister when it is permanently baked on The air dried pieces are then baked in an oven at 230°C, for three hours, after which they are ready for use. Following 150 or more exposures to blood, the previous coating may be removed by treatment with strong alkali These baked-on surfaces are hard and durable. Although they can be scratched, they are not ordinarily damaged by the usual laboratory manipulations

Application of Teflon (for accessible metal surfaces) This plastic may [illegible]

coating

the needle, or inadequate filling of the vein with blood because of defective application of the tourniquet.

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Endothelium (living test tube)	+ generally available	− Jugular vein of dogs & rabbits	− Cost of animal	−	− fragile & permeable	− Semiopaque	− Not recoverable	+ inert	[1, 4]
Plastics rubber	+ generally available	± tubing	+	± *limited*	+ pliable	+ opaque	+ difficult to ensure cleanliness	+ most plastics are inert but impurities may vitiate use entirely Rubber is very unsatisfactory because of (1) substances from curing (2) difficult to ensure clean of blood after use *Others give a series of inert surfaces which can be applied to any situation*[4]	Hales used a goose trachea as a tube Selected representative plastics have been listed The ease of application and wide range of form make the plastics the most useful type of surfaces[1, 4]
polythene	"	"		limited	"		fair		
lusteroid lucite	"	test-tubes Made to specifications		can be machined	rigid	transparent	easily cleaned where access to polishing		
Silicone	Gen Elec Dri-Film 9977 " " 9987	film to apply to all glass surfaces in soln or vapour		can be used to cover any glass surface	−	transparent	See page 9		
Laquer silicone	Gen Elec 81202 Silicone Resin Dow Corning Pan Glaze	permanent surface treatment for accessible stainless steel parts		baked at 230°C for 3 hrs	durable	−	Easily cleaned (life-c 1?0) / Can be removed with strong alkali and reapplied		
Arquad 2C	Armour & Co	solution to apply to needles		supplies surface for needles & other metal parts with narrow diam etc	−	−	removed with soap and water and reapplied each time		
Teflon	Dupont Co	surface coating for metals		can be machined but cheaper as surface applied to metal, baked and polished	rigid		easily cleaned where access to polishing / *		

[1] Hewson, W The Works of Wm Hewson London 1846
[2] Lampert, E Die physikalische Seite des Blutgerinnungsproblem und ihre praktische Bedeutung 127 pp, Leipzig, Georg Thiem, 1931
[3] Pocantins L Relation of contacting surface to coagulation Am J Physiol *143*, 67, 1945
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TABLE 1. *Surfaces for Coatings*

Surface	Availability	Forms	Cost	Machinability Application, etc	Pliability	Transparency	Ease of Cleaning	Restoration of Surface	Effect on Clotting	Remarks and References
Metals Steel aluminum copper brass tin pewter nickel & alloys silver gold platinum	+ generally available	+ needles in steel & noble metals (rare), containers in limited forms	− dependent on metal and amount of machining required	+ chief advantage. Can be accurately machined	± rigid	− opaque, used in taking blood, no visible control in containers observations must be from above	− can be cleaned, where access for scouring and polishing. Difficult to ensure cleanliness in small orifices needles etc. as no visible inspection	? possible where access for polishing. Doubtful where this is not possible	+ noble metals believed inert. Probably true of most metals provided shavings not left from machining and surface polished which is not possible in many uses	Hewson[1] obtained spectacular results with pewter & porcelain containers blood obtained with bistoury.
Glass Soft Hard Quartz	+ generally available	+ containers & tubing in great variety	+ cheap quartz expensive	+ can be worked by any lab worker	± rigid fragile	+ transparent, ease of observation & control	+ easily cleaned	+ rel. easily restored but any variation in techniques may introduce changes in surface	− marked accelerator of clotting. Soft glass believed worse. Quartz inert	?
Paraffin liquid solid vaseline	+ generally available purity?		+ cheap	+ can be applied direct or in solution (benzene) to most glass surfaces	±	+ transparent, ease of observation & control	+ surface is removed by organic solvents & then reapplied each time		+ Inert	
Cellulose *Collodion* Cellophane	+ generally available	+ collodion in solution − cellophane in tubes & sheets	+ cheap	+ collodion can be applied to containers pipettes glass surfaces	~ fragile	+ transparent, ease of observation & control	+	+	+ inert	?

Endothelium (living test tube)	+ generally available	− Jugular vein of dogs & rabbits	− Cost of animal	−	− fragile & permeable	− Semiopaque	− Not recoverable	+ inert	1 4
Plastics rubber	+ generally available	± tubing	+	± limited	+ pliable	+ opaque	+ difficult to ensure cleanliness	+ most plastics are inert but impurities may vitiate use entirely. Rubber is very unsatisfactory because of (1) substances from curing (2) difficult to ensure clean of blood after use *Others give a series of inert surfaces which can be applied to any situation* [4]	Hales used a goose trachea as a tube. Selected representative plastics have been listed. The ease of application and wide range of form make the plastics the most useful type of surfaces.[5 6]
polythene lusteroid lucite	" "	" test-tubes Made to specifications		limited can be machined	" rigid	transparent	fair easily cleaned where access to polishing		
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Arquad 2C	Armour & Co	solution to apply to needles		supplies surface for needles & other metal parts with narrow diam etc	−	−	removed with soap and water and reapplied each time		
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3. Preparation of Glassware

L. B. JAQUES

All glassware should be first washed thoroughly with soap and water, rinsed repeatedly with tap water, and after rinsing with distilled water, dried and then inspected Any showing cracks and scratches must be discarded. Detergents should not be used for washing, since these have a harmful effect on leucocytes.

Further treatment depends on the measurement being made. Many procedures require treatment with chromate-sulphuric acid cleaning mixture. This removes fat by oxidation but will fix protein in situ. Also, it is very difficult to remove the traces of chromic acid. Treatment with chromate cleaning solution is done in one of two ways. Some prepare the cleaning solution by pouring carefully 1 liter of concentrated sulphuric acid into 35 ml. saturated sodium dichromate. The glassware is allowed to sit in this overnight. Others prepare a diluted solution of sodium dichromate-sulphuric acid Articles are boiled in this for 30 minutes.

Alternative procedures for final preparation of glassware recommended by different workers are

1 treatment with chromate-sulphuric cleaning solution, followed by rinsing 20 times with distilled water,

2 treatment with chromate-sulphuric acid cleaning solution, rinsing in tap-water, boiling in distilled water, rinsing again with distilled water,

3. treatment with chromate-nitric acid and extensive rinsing,

4 treatment with 40 per cent NaOH, rinsing, treatment with chromate cleaning solution, extensive rinsing,

5 after washing with soap and water, the glassware is rinsed thoroughly with distilled water, then rinsed twice with alcohol, twice with ether and dried,

6. after washing with soapy water, the glassware is rinsed thoroughly with tap water, then distilled water and dried.

It is extremely important in the handling of ordinary glassware to avoid completely all contact with silicone (see p 10).

4. Removal and Application of Silicone

L. M. TOCANTINS

Siliconized glassware (pipettes, tubes, syringes) should be cleaned by first washing in warm water then immersing the glassware in the following mixture:

Technical grade acetone	2 liters
H_2O	2 liters
NaOH (solid)	200 Gm

Dissolve the NaOH in about 300 ml. of H_2O before adding to the acetone and H_2O Allow the glassware to remain in the solution overnight, then remove the glassware, rinse thoroughly with H_2O and dry in the oven. Alternatively the well rinsed glassware may be immersed in a solution of equal parts of technical grade acetone and 5.25 per cent sodium hypochlorite with 25 grams NaOH per liter of solution. Eight hours are necessary to remove the silicone. The glassware is then removed from this solution, rinsed well (a brush may be necessary in some instances) and dried in the oven.

SILICONIZING

A solution of 1 part Dri-Film [SC-87] (General Electric) and 4 parts of petroleum ether is used to coat the glass surfaces, which must be clean and dry. Technic

Test Tubes One tube is filled to the top with the silicone-petroleum ether solution, the contents poured into a second, from the second to the third and so on until all are finished As the solution is poured from one tube to another it is rotated, so that the entire inner surface of the tube is coated with the silicone When the tubes are all coated they are filled with distilled water, allowed to stand from 15 to 30 minutes, drained, rinsed thoroughly with distilled water and inverted and put in the oven to dry at 120°C from 60 to 90 minutes. They are then tightly covered, dated and stored in a clean, dry closet until used

Pipettes. Draw the solution up into the pipette with a small rubber bulb dipping the pipette about two inches into the solution (so as to siliconize the outer surface) Draw the solution only about an inch below the mouth end Drain the pipettes well and rinse thoroughly with distilled water and place in oven (120°C) to dry for about 60 minutes

Syringes A few cc of the solution are drawn into the syringe which is then inverted and the plunger pulled down slowly as the silicone coats the barrel sides This is repeated two or three times so as to coat the plunger

The silicone solution is then forced out, the barrel and plunger separated and thoroughly rinsed with distilled water and placed in the oven to dry. Do not put syringe together until completely dry.

Needles are siliconized in the same manner and at the same time. Merely place the needle on the syringe tip at the beginning of the process before aspirating the silicone solution.

Special precautions should be taken to avoid all contact of siliconized glassware with non-siliconized stock. This requires separate cleaning and storage facilities.

5. List of Anticoagulants Employed in Studies on Blood Coagulation

R. R. HOLBURN

No	Compound	Formula	Per Cent Solution	Preparation	Vol. of Solution to Vol. of Blood
1	Sodium Citrate	$C_6H_5Na_3O_7 \cdot 2H_2O$	3.2 3.8 19.0 38.0	3.2 Gm./100 ml. H_2O 3.8 Gm./100 ml. H_2O 19 Gm./100 ml. H_2O 38 Gm./100 ml. H_2O	1 to 9 1 to 9 1 to 49 1 to 99
2	Sodium Oxalate	$Na_2C_2O_4$	1.34	1.34 Gm./100 ml. H_2O	1 to 9
3	Potassium Oxalate	$K_2C_2O_4$	1.4	1.4 Gm./100 ml. H_2O	1 to 9
4	Lithium Oxalate	$Li_2C_2O_4$	1.4	1.4 Gm./100 ml. H_2O	1 to 9
5	Ammonium Oxalate*	$(NH_4)_2C_2O_4 \cdot H_2O$	1.4	1.4 Gm./100 ml.	1 to 9
6	Dowex 50 Na† Amberlite IRC 50†	cationic exchanger cationic exchanger		50 Gm. 35 Gm.	500 ml. 500 ml.
7	Oxalate Mixture Ammonium Oxalate Potassium Oxalate	 $(NH_4)_2C_2O_4$ $K_2C_2O_4$	2.0	 1.2 Gm. + 0.8 Gm./100 ml. H_2O	1 to 9
8	Mixture Potassium Oxalate Sodium Fluoride	 $K_2C_2O_4$ NaF	20.0	 10 Gm. 10 Gm./100 ml. H_2O	1 to 19
9	Heparin	—	1.0	1 Gm./100 ml. H_2O	1 to 9
10	Disodium sequestrene	$C_{10}H_{14}N_2Na_2O_8$	1.0	1 Gm./100 ml. H_2O	1 to 9
11	Sodium Fluoride	NaF	40.0	40 Gm./100 ml. H_2O	1 to 99
12	Potassium Fluoride	$KF \cdot 2H_2O$	40.0	40 Gm./100 ml. H_2O	1 to 99
13	A C D Solution*	N I H, Formula B	3.23	0.44 Gm. citric acid, (Anhydrous), U S P 1.32 sodium citrate U S P 1.47 Gm. dextrose (hydrous), U S P	1 to 4

* U S Dispensatory, 25th ed. Eds. A. Osol and G. E. Farrar, Jr., 1955, p. 1271.
† Denkewalter, R. C. and Kazal, L. A. Chap. 20 in Ion Exchange Technology. F. C. Nachod and J. Schubert, eds. New York, Academic Press, 1956, pp. 592-6.

The silicone solution is then forced out, the barrel and plunger separated and thoroughly rinsed with distilled water and placed in the oven to dry. Do not put syringe together until completely dry.

Needles are siliconized in the same manner and at the same time. Merely place the needle on the syringe tip at the beginning of the process before aspirating the silicone solution.

Special precautions should be taken to avoid all contact of siliconized glassware with non-siliconized stock. This requires separate cleaning and storage facilities.

For 0 2 ionic strength buffers, 72 ml. of 5.0 M NaCl is added to the mixtures and diluted to 2 liters to give the desired pH. For 0 1 ionic strength buffers, 32 ml. of 5 0 M NaCl is added and diluted to 2 liters with distilled water.

C. Veronal (barbital) buffer:

Stock solutions:
2 0 N HCl
0 5 M barbital sodium (sodium diethylbarbiturate)
5.0 M NaCl

pH	7 5	8 0	8 5	9 0
ml. 2 0 N HCl .	14 4	10 4	5 3	2 0
ml. 0 5 M barbital . .	80 0	80 0	80 0	80 0

For 0 2 ionic strength buffers, 72 ml. of 5 0 M NaCl is added to the mixtures and diluted to 2 liters to give the desired pH. For 0 1 ionic strength buffers, 32 ml. of 5.0 M NaCl is added and diluted to 2 liters with distilled water

Reference

G L. Miller and Golder R H Buffers of pH 2 to 12 for use in electrophoresis. Arch Biochem 29 420, 1950

D Borate buffer, pH 7.7

45 volumes of 2.5% H_3BO_3
45 volumes of 0 5% NaCl
10 volumes of 4 0% $Na_2B_4O_7 \cdot 10H_2O$

Reference

J H Ferguson, B L.Travis and E Gerheim: The activation of prothrombin, with special reference to "thromboplastic enzyme" (tryptase) Blood 3 1130, 1948

6. Buffer Solutions for Studies on Blood Coagulation

R. R. HOLBURN

A. Imidazole Buffer-pH range 6.2—7.8 (Mertz and Owen).

Prepare the following stock solutions:

1. 0.1 N HCl (adjust pH to 1.08 ± 0.1)
2. 0.2 M Imidazole solution. Dissolve 1.36 Gm of Imidazole powder (Eastman Kodak, Rochester, N.Y.) in distilled H_2O to a total volume of 100 ml.

In order to obtain solutions of the following pH, the corresponding amounts stated below must be mixed and diluted with H_2O to a volume of 100 ml.

pH	6.2	6.4	6.6	6.8	7.0	7.2	7.4	7.6	7.8
ml. of Stock HCl Solution	42.9	39.8	35.5	30.4	24.3	18.6	13.6	9.3	6.0
ml of Stock Imidazole Solution	25	25	25	25	25	25	25	25	25

After mixture, the pH of each solution should be checked with a pH meter

REFERENCE

Mertz, E. T. and Owen, C. A. Imidazole buffer. Its use in blood clotting studies. Proc. Soc. Exper. Biol. & Med. 43: 204, 1940

B. Sodium phosphate buffer:

Stock solutions.
0.5 M Na_2HPO_4
4.0 M NaH_2PO_4
5.0 M NaCl

pH	6.0	6.5	7.0	7.5
ml 0.5M Na_2HPO_4	9.2	16.6	22.7	24.3
ml 4.0M NaH_2PO_4	6.6	3.7	1.6	0.5

CHAPTER II

INTRODUCTORY EXERCISES

L. M. TOCANTINS and R. R. HOLBURN

In this chapter are grouped what may be called the calisthenics for beginners in work on blood coagulation. The exercises are designed to acquaint the neophyte, or near-neophyte, with the language and facts of the subject. Since they have not been arranged in the order of simplicity, it may be wise if the inexperienced worker selects the simplest exercise and progresses gradually to the more complex ones. It is the custom in our laboratory to have a technical worker spend from two to three months performing these exercises and submitting the data to the senior workers in the department. It is only after such a period of apprenticeship that we feel the technical worker may become sufficiently acquainted with the fundamental facts of blood coagulation to render his (or her) help effective and valuable. At this stage of their training, it is insisted that they do not read texts or articles on blood coagulation This is for the purpose of providing them with a stock of knowledge of these facts before they are plunged into the treacherous currents of the theory and terminology of blood coagulation.

EXERCISE No 1: *Effect on Clotting Time of Changing the Volume of the Blood While Maintaining the Diameter of the Tube Constant*

Five uncoated glass and five silicone-coated tubes of 13 mm i d. were selected. To each group of 5 was added: 0.5, 1 0, 1.5, 2.0 and 3.0 ml of blood from a normal subject, collected with the silicone technic. The tubes were stoppered and kept at 38°C

Results:

Tube No	1	2	3	4	5
Amount of Blood (ml)	0 5	1 0	1 5	2 0	3 0
Clotting Time (mins)					
Glass	10	15	16 5	21	21 5
Silicone	20	35	36	40	42

The rate of blood coagulation, in glass or silicone coated surfaces, increases as the volume of the blood is increased

7. Estimation of Per Cent Concentration of Plasma in a Clotting Mixture

L. M. TOCANTINS

The actual concentration of plasma separated from a sample of citrated (or oxalated) blood and the final plasma concentration in a clotting mixture may be determined as follows: Measure the hematocrit in the citrated blood. Estimate the concentration of the removed plasma making allowance for the volume of the anticoagulant added. To determine the plasma concentration in the clotting mixture divide the volume of plasma by the total volume of the mixture and multiply by the citrated plasma concentration

Example:

Hematocrit = 37 on blood collected with one part 38 per cent sodium citrate to 99 parts of blood. Sixty-three per cent of the blood sample is therefore plasma. In the 63 parts of the plasma, there is one part of citrate solution Therefore in order to calculate the actual plasma concentration in the original sample:

$$\frac{62}{63} \times 100 = 98.4\% \text{ plasma concentration}$$

In order to calculate the final concentration of plasma in a clotting mixture of 0.35 ml plasma, 0.165 ml. 0.85 per cent NaCl, 0.035 ml 0.2 M $CaCl_2$:

$$\frac{\text{Plasma vol}}{\text{Total vol}} \times 98.4 = \frac{35}{55} \times 98.4$$

$$= 62.6\% \text{ plasma concentration in clotting mixture}$$

Results:

Tube No	1	2	3	4	5
pH	6 4	6 8	7 2	7 5	7 9
Clotting Time (secs.)	29	26	19	22	30

Raising or lowering of pH beyond 7.2 slows the rate of clotting of activated plasma.

EXERCISE No. 5: *Effect of Concentration of NaCl on Clotting Time of Nonactivated Plasma*

Clotting Mixture: 0 1 ml NaCl solution (variable per cent concentration).
0.1 ml. Human Citrated Plasma.
0.1 ml 0 02 M $CaCl_2$

Glass tubes, 38°C.

Results:

Tube No	NaCl Solution (% Concentration)	Clotting Time secs)
1	0 01	520
2	0 025	515
3	0 05	510
4	0 10	500
5	0 25	475
6	0 5	460
7	0 85	430
8	0 95	490
9	1 0	620
10	1 5	925
11	2 0	1,180
12	8 5	>21,600

NaCl solution of 0 85 per cent concentration seems to supply the optimal conditions for coagulation

EXERCISE No 6 *Effect of Different Surfaces on Rate of Coagulation and Clot Retraction of Normal Blood*

Blood was collected with the silicone technic and 1 ml was placed in each tube with a surface as indicated below

Type of Surface	Glass	Paraffin	Collodion	Silicone
Clotting Time (mins)	6½	15	17	28
Serum expressed in 2 hours (% of clot volume)	32	40	0	50

EXERCISE NO. 2: *Effect on the Rate of Blood Coagulation of Changing the Diameter of the Tube While Maintaining the Volume of the Blood Constant*

Four uncoated glass and four silicone-coated tubes of the following internal diameter: 7 mm, 10 mm., 13 mm., 15 mm., were selected. To each was added 1 ml. of blood from a normal subject, collected with the silicone technic. The tubes were stoppered and kept at 38°C.

Results:

Tube No	1	2	3	4
Internal Diameter (mm) . .	7	10	13	15
Clotting Time (mins.)				
Glass.. .. .	14	19	20	23
Silicone	30	32	34	38

The greater the diameter of the tubes the longer the clotting time in uncoated glass tubes. In silicone-coated tubes, clotting time is less influenced by increase in diameter of the tube.

EXERCISE NO. 3. *Effect of pH on Clotting Time of Nonactivated Plasma*

Clotting Mixture: 0.1 ml. Imidazole Buffer (var. pH)
0.1 ml. Human Plasma
0.1 ml. 0.02 M $CaCl_2$

Glass Tubes, 38°C

Results:

Tube No	pH of Buffer Solution	Clotting Time (secs)
1	6 0	490
2	6 4	378
3	6 8	242
4	7 2	179
5	7 5	168
6	7 9	330
7	8 2	504

EXERCISE NO. 4 *Effect of Change in pH on the Clotting Time of Activated Plasma*

Clotting Mixture, 0.1 ml. Imidazole Buffer (variable pH)
0.1 ml. Human Brain Thromboplastin
0.1 ml. 0 02 M $CaCl_2$
0 1 ml Human Plasma

Glass tubes, 38°C.

The solutions of imidazole are prepared as described on page 12.

Results:

Tube No	1	2	3	4	5
pH	6 4	6 8	7 2	7.5	7.9
Clotting Time (secs)	29	26	19	22	30

Raising or lowering of pH beyond 7.2 slows the rate of clotting of activated plasma.

EXERCISE No 5: *Effect of Concentration of NaCl on Clotting Time of Nonactivated Plasma*

Clotting Mixture: 0.1 ml. NaCl solution (variable per cent concentration).
0.1 ml. Human Citrated Plasma.
0.1 ml. 0 02 M $CaCl_2$

Glass tubes, 38°C.

Results:

Tube No	NaCl Solution (% Concentration)	Clotting Time secs)
1	0 01	520
2	0 025	515
3	0 05	510
4	0 10	500
5	0 25	475
6	0 5	460
7	0 85	430
8	0 95	490
9	1 0	620
10	1 5	925
11	2 0	1,180
12	8 5	>21,600

NaCl solution of 0 85 per cent concentration seems to supply the optimal conditions for coagulation.

EXERCISE No 6: *Effect of Different Surfaces on Rate of Coagulation and Clot Retraction of Normal Blood*

Blood was collected with the silicone technic and 1 ml was placed in each tube with a surface as indicated below.

Type of Surface	Glass	Paraffin	Collodion	Silicone
Clotting Time (mins)	6½	15	17	28
Serum expressed in 2 hours (% of clot volume).	32	40	0	50

Rate of clotting was fastest in glass and slowest in silicone tubes.

Clot retraction was greatest in silicone and paraffin, less in glass and absent in collodion.

REFERENCE

Lozner, E. L. and Taylor, F. H. L.: Foreign surfaces and blood coagulation. J. Clin Invest *21*: 241, 1942.

EXERCISE NO. 7: *Effect of Using the Same Syringe on 2 Successive Determinations of the Venous Blood Clotting Time*

Blood was collected from a normal subject using a freshly coated siliconized syringe. It was then placed in three 13 mm. i.d. siliconized tubes, as indicated below, and the clotting time determined at 38°C. The syringe was then rinsed with 0 85 per cent NaCl several times, dried and used again as before. Blood was collected from another vein of the same subject.

Results:

Tube No ...	1	2	3
Blood (ml)	1	0 6	0 3
0 85% NaCl (ml.)	0	0 4	0 7
Per cent conc of the blood	100	60	30
Clotting Time (mins)			
(a) fresh syringe	34	22	32
(b) once used syringe	23	19	27

A significant shortening of the clotting time results when the same syringe is used for 2 determinations, without recoating the surface of the syringe

REFERENCE

Tulloch, J A, Overman, R S and Wright, I S: Failure of ingestion of cream to affect blood coagulation Am J Med *14* 674, 1953

EXERCISE NO 8 *Effect of Using the Same Tube on 2 Successive Determinations of the Plasma Clotting Time*

Normal citrated human plasma was collected with the silicone technic 0.5 ml of the plasma was then pipetted into one uncoated and one silicone-coated glass tube 13 mm. i.d , 0 05 ml 0 2 M $CaCl_2$ was added to each and the clotting time measured at 38°C The clots were removed from the tubes 1 5 minutes after they were formed and then the tubes were rinsed 5 times with 0 85 per cent NaCl, inverted and allowed to dry. 0 5 ml. of the plasma was then introduced into each tube, recalcified as above and the clotting time measured.

Results:

Tube No	1	2
Surface	Glass	Silicone
Clotting Time (secs.)		
Before rinsing	969	1495
After rinsing	604	1220

Repeated use of the same tube, without proper processing, tends to accelerate the clotting times of the plasma, especially when uncoated glass tubes are employed.

EXERCISE No. 9: *Effect of Varying the Temperature on the Rate of Coagulation of Normal Platelet-Poor Plasma*

Citrated plasma was placed in 13 mm. wide silicone-coated tubes and kept in a constant temperature water bath for 3 minutes before recalcification

Clotting Mixture: 0.1 ml. 0 85% NaCl
0.1 ml. Plasma
0.1 ml 0 02 M $CaCl_2$

Results:

Tube No	1	2	3	4	5	6	7	8
Temperature (degree C)	20	25	30	35	38	40	45	50
Clotting Time (secs)	1940	1398	1020	775	560	588	875	1010

The optimum temperature for coagulation is near 38°C Temperatures of 35° or below or 45° or above delay clotting

EXERCISE No 10 *Effect of Freezing and Thawing on the Clotting Time of Plasma*

Normal and hemophilia A platelet-poor plasmas were collected with special precautions using 19 per cent Na citrate as anticoagulant Aliquots of 0 5 ml plasma were placed in siliconized test tubes For quick freezing, the test tubes were immersed in an acetone-dry ice bath, then stored at −20°C For slow freezing, the samples were placed in the freezer at −20°C. For quick thawing, the samples were placed under running hot water, 55-60°C., until just fluid Slow thawing took place at 37°C To test the clotting time, 0 05 ml. 0 2 M $CaCl_2$ was added to each sample Clotting temperature: 37°C.

Control clotting time of plasma:

Normal	1350″
Hemophilia A	No clot

Number of Times Frozen and Thawed	Quick Freezing				Slow Freezing			
	Quick thawing		Slow thawing		Quick thawing		Slow thawing	
	Norm	Hemo.	Norm.	Hemo.	Norm.	Hemo.	Norm.	Hemo.
First...	890″	No clot	970″	No clot	710″	3215″	685″	4200″
Second...	755″	No clot	890″	13,500″	700″	2000″	680″	4250″
Third. ...	700″	No clot	900″	11,500″	700″	1400″	680″	2300″
Fourth.....	700″	No clot	900″	11,000″	700″	1400″	500″	1400″

Freezing plasma shortens the clotting time. Quick freezing and quick thawing has the least effect on clotting time.

EXERCISE NO. 11: *Effect of Incubation at Various Temperatures on the Clotting Time of Plasma*

Normal platelet-poor citrated plasmas were collected with special precautions. Nine parts of plasma were mixed with one part of buffered physiologic salt solution Incubation was carried out in new plastic (polystyrene) tubes.

Clotting Mixture siliconized test tubes, 37°C.
0 2 ml. plasma
0 02 ml 0.2 M $CaCl_2$

Temperature	Period of Incubation					
	0 hr	1 hr	2 hr	3 hr	5 hr	24 hr.
4°C	1570″	1350″	1650″		1250″	2450″
24°C.	1200″	1200″		1225″	1290″	>10,000″
37°C.	550″	780″	1075″	1020″	1175″	

Incubation of plasma at temperatures of 25°C. or less results in minimal changes in clotting activity over a 5-hour period. After 24 hours, *there is considerable loss in clotting activity.* At 37°C, the loss in clotting activity is more rapid.

EXERCISE NO 12: *Effect of Tilting Movements on Clotting Time in Glass and Silicone Tubes*

Two sets of 13 mm. diameter tubes were arranged, 5 glass and 5 silicone-coated Eleven ml. of blood were drawn and 1 ml placed in each of the 10 tubes, in a water bath at 38°C The clotting time of the blood in No. 1 tube was determined. After the blood in No. 1 had coagulated, the clotting time in No. 2 was done, etc. The tubes were not disturbed until the blood in the preceding tube had coagulated

Results:

	Clotting Time in Seconds				
	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5
Glass Tubes................	440	600	750	900	1050
Silicone Tubes....	775	1250	1450	1650	1850

Tilting the tube to detect the end point in clotting hastens coagulation in both glass and silicone-coated tubes.

Exercise No 13: *Effect of Multiple Venous Punctures on Clotting Time of Blood*

Blood was collected with the silicone technic from a normal person and a hemophilic patient with poor veins. In the first venepuncture, several attempts were made to obtain blood before the needle entered the vein In the second venepuncture, another vein was easily found, the first few ml. of blood discarded and blood for the clotting times collected with a fresh syringe. Silicone tubes and syringes. Temperature, 38°C.

Results:

Tube No.	Contents	Concentration of blood (per cent)	Clotting Times (mins.)			
			1st puncture		2nd punture	
			Normal	Hemophilic	Normal	Hemophilic
1	1 0 *ml* blood	100	3½	8½	33	188
2	0 6 ml blood 0.4 ml. 0 85% NaCl	60	5	16	18	150
3	0 3 *ml* blood 0 7 ml. 0 85% NaCl	30	8	20	21	100

Repeated trial at vein puncture: (1) Accelerates the coagulation of both normal and hemophilic blood, especially the latter (2) Eliminates the clot accelerating effect produced by dilution in both types of blood (3) Also reduces or eliminates the difference between the rate of coagulation of blood in glass and silicone-coated surfaces (not shown in table)

Exercise No 14: *Effect of Varying the Concentrations of $CaCl_2$ on the Clotting Time of Nonactivated Plasma*

This exercise was performed with the same plasma used in the following exercise. Instead of thromboplastin, 0 85 per cent NaCl was used in the clotting mixture.

Clotting Mixture: 0.1 ml. 0.85% NaCl
0 1 ml. Plasma
0.1 ml $CaCl_2$ (variable M concentration)

Results:

Tube No.	1	2	3	4	5	6	7	8	9	10
Molar Conc. $CaCl_2$....	1	25	.125	065	03	025	02	015	.008	004
Clot Time (secs)	>5000	>5000	>5000	2160	392	390	317	482	>5000	>5000

As in the activated plasma clotting mixture, a solution of 0.02 M concentration provides the optimum amount of $CaCl_2$. The nonactivated mixture is even more sensitive to excessive or insufficient recalcification than those containing thromboplastin. Variations in hematocrit will, therefore, be more promptly reflected in this than in the activated plasma clotting mixtures.

EXERCISE NO. 15: *Effect of Varying the $CaCl_2$ Concentration on the Clotting Time of Activated Plasma*

$CaCl_2$ solutions of various molar concentration were prepared. Using normal plasma and human brain thromboplastin the clotting time was measured in mixtures containing variable amounts of $CaCl_2$. The plasma was derived from blood to which trisodium citrate was added (1 part of a 3.8 per cent solution of citrate to 9 parts of blood) Since the hematocrit was 40 per cent, 38 mg. of citrate were contained in 6 ml of plasma or a citrate concentration of 0 63 per cent.

Clotting Mixture. 0 1 ml. Thromboplastin
0.1 ml $CaCl_2$ (variable molar concentration)
0 1 ml. Plasma

Temperature 38°C, glass tubes.

Results

Tube No .	1	2	3	4	5	6	7	8	9	10
Molar Conc. $CaCl_2$	1	25	125	065	03	025	02	015	008	004
Clotting Time (secs)	>5000	451	34	23	19	17	15	17	18	65

A 0 02 M solution of $CaCl_2$ supplies the optimal recalcification for plasmas with a citrate *concentration such as that of the plasma tested If* a 3.8 per cent solution is used and the proportions of citrate solution to

blood (1–9) are maintained, the optimum amount of calcium needed does not vary significantly provided the hematocrit is 40% ± 5. A high hematocrit will raise the citrate concentration of the plasmas, while a low one will do the opposite, therefore altering the amount of $CaCl_2$ required for recalcification

EXERCISE No. 16: *Effect of Dilution on the Rate of Clotting of Plasma*

Fifteen ml. of stable platelet poor normal plasma were separated from blood collected with a siliconized syringe containing 0.2 ml. 19 per cent Na citrate per 10 ml. blood. The original plasma concentration, taking 37 as the hematocrit, would then be 98 per cent. Temperature, 38°C.

Results:

Tube No	Contents	Conc of Plasma (%)	Clotting Time (secs)	
			Glass	Silicone
1	0 5 ml Plasma 0 05 ml. 0 2 M $CaCl_2$	89	360	1440
2	0 35 ml Plasma 0 165 ml 0 85% NaCl 0 035 ml 0 2 M $CaCl_2$	63	350	705
3	0 16 ml Plasma 0 23 ml 0 85% NaCl 0 16 ml 0 02 M $CaCl_2$	29	180	305
4	0 08 ml Plasma 0 39 ml 0 85% NaCl 0 08 ml 0 02 M $CaCl_2$	14	270	445
5	0 04 ml Plasma 0 47 ml 0 85% NaCl 0 04 ml 0 02 M $CaCl_2$	7	1040	1030
6	0 01 ml. Plasma 0 53 ml 0 85% NaCl 0 01 ml 0 02 M $CaCl_2$	2	>3600	>3600

Dilution shortens the rate of coagulation of plasma and eventually equalizes the difference in the rate of clotting between plasma in glass and silicone-coated tubes

REFERENCE

Tocantins, L. M., Holburn, R R., Carroll, R T and Stoker, J W The rate of coagulation of the blood and plasma in contact with glass, silicone and other surfaces Trans of the 3rd Conference on Blood Clotting of the Macy Foundation, page 127, 1950

Clotting Mixture: 0.1 ml 0.85% NaCl
0.1 ml. Plasma
0.1 ml $CaCl_2$ (variable M concentration)

Results:

Tube No.... . .	1	2	3	4	5	6	7	8	9	10
Molar Conc $CaCl_2$.. .	1	25	125	065	03	025	.02	.015	.008	004
Clot Time (secs)	>5000	>5000	>5000	2160	392	390	317	482	>5000	>5000

As *in the activated plasma clotting mixture, a solution of 0.02 M* concentration provides the optimum amount of $CaCl_2$. The nonactivated mixture is even more sensitive to excessive or insufficient recalcification than those containing thromboplastin. Variations in hematocrit will, therefore, be more promptly reflected in this than in the activated plasma clotting mixtures

EXERCISE No. 15 *Effect of Varying the $CaCl_2$ Concentration on the Clotting Time of Activated Plasma*

$CaCl_2$ solutions of various molar concentration were prepared. Using normal plasma and human brain thromboplastin the clotting time was measured in mixtures containing variable amounts of $CaCl_2$. The plasma was derived from blood to which trisodium citrate was added (1 part of a 3.8 per cent solution of citrate to 9 parts of blood) Since the hematocrit was 40 per cent, 38 mg of citrate were contained in 6 ml of plasma or a citrate concentration of 0 63 per cent

Clotting Mixture. 0 1 ml Thromboplastin
0 1 ml $CaCl_2$ (variable molar concentration)
0.1 ml Plasma

Temperature 38°C, glass tubes.

Results:

Tube No	1	2	3	4	5	6	7	8	9	10
Molar Conc. $CaCl_2$	1	25	125	065	03	025	02	015	008	004
Clotting Time (secs.). .	>5000	451	34	23	19	17	15	17	18	65

A 0.02 M solution of $CaCl_2$ supplies the optimal recalcification for plasmas with a citrate concentration such as that of the plasma tested If a 3 8 per cent solution is used and the proportions of citrate solution to

blood (1–9) are maintained, the optimum amount of calcium needed does not vary significantly provided the hematocrit is 40% ± 5. A high hematocrit will raise the citrate concentration of the plasmas, while a low one will do the opposite, therefore altering the amount of $CaCl_2$ required for recalcification

EXERCISE No. 16: *Effect of Dilution on the Rate of Clotting of Plasma*

Fifteen ml. of stable platelet poor normal plasma were separated from blood collected with a siliconized syringe containing 0.2 ml 19 per cent Na citrate per 10 ml blood The original plasma concentration, taking 37 as the hematocrit, would then be 98 per cent. Temperature, 38°C.

Results·

Tube No	Contents	Conc. of Plasma (%)	Clotting Time (secs)	
			Glass	Silicone
1	0 5 ml Plasma 0 05 ml 0 2 M $CaCl_2$	89	360	1440
2	0 35 ml. Plasma 0 165 ml 0 85% NaCl 0 035 ml 0 2 M $CaCl_2$	63	350	705
3	0 16 ml. Plasma 0 23 ml 0 85% NaCl 0 16 ml 0 02 M $CaCl_2$	29	180	305
4	0 08 ml Plasma 0 39 ml 0 85% NaCl 0 08 ml 0 02 M $CaCl_2$	14	270	445
5	0 04 ml Plasma 0 47 ml 0 85% NaCl 0 04 ml 0 02 M $CaCl_2$	7	1040	1030
6	0 01 ml Plasma 0 53 ml 0 85% NaCl 0 01 ml 0 02 M $CaCl_2$	2	>3600	>3600

Dilution shortens the rate of coagulation of plasma and eventually equalizes the difference in the rate of clotting between plasma in glass and silicone-coated tubes

REFERENCE

Tocantins, L M, Holburn, R R, Carroll, R T and Stoker, J W The rate of coagulation of the blood and plasma in contact with glass, silicone and other surfaces Trans of the 3rd Conference on Blood Clotting of the Macy Foundation, page 127, 1950

EXERCISE No. 17: *Effect of Dilution on Clotting Time of Activated Plasma*
Same Plasma Used in Previous Exercise

Initial plasma concentration: 98 per cent, Temperature 38°C.

Results:

Tube No	Contents	Conc. of Plasma (%)	Clotting time (secs)	
			Silicone	Glass
1	0 5 ml Plasma 0 1 ml Thrombopl. 0 05 ml 0 2 M $CaCl_2$	74	22	20
2	0 35 ml Plasma 0 165 ml. 0 85% MaCl 0 1 ml. Thrombopl 0 035 ml. 0 2 M $CaCl_2$	52	20	19
3	0 16 ml Plasma 0 23 ml 0 85% NaCl 0 1 ml Thrombopl 0 16 ml. 0 02 M $CaCl_2$	24	18	18
4	0 08 ml Plasma 0 39 ml 0 85% NaCl 0 1 ml. Thrombopl 0 08 ml 0 02 M $CaCl_2$	12	23	23
5	0 04 ml Plasma 0 47 ml 0 85% NaCl 0 1 ml Thrombopl 0 04 ml 0 02 M $CaCl_2$	6	42	42
6	0 02 ml Plasma 0 51 ml 0 85% NaCl 0 1 ml Thrombopl 0 02 ml 0 02 M $CaCl_2$	3	72	71

REFERENCE

Tocantins, L M, Carroll, R T and Holburn, R R Influence of the final plasma concentration in the actual clotting mixture on the response of normal and hemophilic plasmas to thromboplastin Trans of the 3rd Conference on Blood Clotting and Allied Problems of the J Macy, Jr Foundation, 1950, p 192

EXERCISE No. 18 *Effect of Centrifugation of Normal Blood on Rate of Clotting of Plasma*

Fifty ml of citrated blood collected with the usual precautions were centrifuged at 1000 rpm for various lengths of time as listed below. When a sample was to be taken, 1 5 ml of plasma from the upper portion was removed by a capillary dropper and placed into a separate tube The re-

maining sample was centrifuged again for the periods stated below. Care was taken not to disturb the buffy layer when aspirating the plasma.

Clotting Mixture: 0.5 ml. plasma
0.05 ml. 0.2 M $CaCl_2$

Results:

Duration of Centrifugation (mins.)	Total Centrifugation (mins.)	Clotting Time (secs.)
8	8	960
7	15	1030
10	25	1140
20	45	1590
45	90	2100
90	180	2300

When testing samples in duplicate or triplicate, the three tubes are held at once and tilted at the same time to detect the end point of clotting.

If a platelet count is done on each plasma sample, a relationship will be seen to exist between the number of platelets and the duration of the centrifugation. The longer and faster the centrifugation (the greater the g) the less platelets will be in the supernatant plasma and the longer the clotting time.

EXERCISE NO. 19: *Effect of Centrifugation on Rate of Clotting of Normal Plasma*

Twenty ml. of platelet poor normal plasma were separated from properly collected blood centrifuged in silicone-coated tubes for ½ hour at 1000 rpm. The plasma was divided into five tubes and each of the tubes was centrifuged at 5°C. in a horizontal head for the periods stated below. The upper half of each plasma specimen was then removed and tested. Silicone surfaces, 38°C.

Clotting Mixture: 0.5 ml. plasma
0.05 ml. 0.2 M $CaCl_2$

Results

Time of Centrifugation: (mins.) . . .	15	30	60	120	240
Clotting Time (secs.)	710	2650	8610	17 000	>20,000

Essentially incoagulable plasma may be obtained by sufficiently prolonged centrifugation, provided an exacting technic is used in the collection, separation, measuring and testing of the plasma.

EXERCISE No. 20: *Effect of Standing in a Plain and in a Silicone-Coated Glass Vessel on the Rate of Coagulation of Plasma*

Ten ml. of stable normal citrated plasma were collected with the silicone technic and 1 ml. aspirated into each of the ten 1 ml. pipettes, graduated in 0.01 ml. Five of the pipettes were of plain glass and five were of silicone-coated glass. The filled pipettes were allowed to stand at room temperature (18–20°C.) for the periods of time indicated below, before testing plasma at 38°C. in silicone-coated tubes.

Clotting Mixture: 0 5 ml. plasma
0.05 ml. 0 2 M $CaCl_2$

Results.

Duration of Standing (mins)	0	5	15	30	60
Clotting Time (secs)					
Glass	2250	1510	1365	1150	410
Silicone	2280	2170	2270	2310	2270

The clotting time of plasma is markedly shortened by short periods of standing in uncoated glass vessels but little affected when in contact with silicone-coated surfaces.

REFERENCE

Tocantins, L M Influence of the contacting surafce on the coagulability and anti-cephalin activity of normal and hemophilic plasmas Am J Physiol *143* 67-76, 1945

EXERCISE No. 21 *Effect of Tissue Thromboplastins from Different Species on Rate of Coagulation of Plasma*

Materials· (1) Citrated plasma from rabbit, mouse and man (2) Thromboplastin prepared by the method described on page 148 using acetone dried (a) human (b) rabbit and (c) mouse brain.

Clotting Mixture 0 1 ml plasma
0 1 ml thromboplastin
0 1 ml 0 02 M $CaCl_2$

Results:

Source of Thromboplastin	Clotting Time (Seconds)		
	Human Plasma	Rabbit Plasma	Mouse Plasma
Human brain	12	14	16
Rabbit brain	12	7	13
Mouse brain	72	22	9

Each thromboplastin seems to be most effective when tested on homologous plasma

EXERCISE No 22: *Effect of Addition of Thromboplastin of Variable Concentration on Clotting Time of Plasma*

Clotting Mixture: 0.1 ml. human brain thromboplastin (variable dilution).
0 1 ml. 0 02 M $CaCl_2$
0.1 ml. human citrated plasma

In this mixture the plasma concentration is about 26 per cent.

Dilution of the thromboplastin made with 0 85 per cent NaCl immediately before use.

Results

Tube No	Concentration of Thromboplastin (per cent)	Clotting Time (secs)
1	100	21
2	50	22
3	25	22
4	12 5	25
5	10	25
6	5	30
7	2 5	34
8	1 0	78
9	0 5	120
10	0 25	190
11	0 125	254

EXERCISE No 23 *Effect on the Clotting Time of Varying the Concentration of the Plasma but Maintaining that of Thromboplastin and $CaCl_2$ Constant*

Normal and hemophilic human citrated plasma collected with especial precautions Human brain thromboplastin as activating agent.

13 mm. i d Silicone tubes, 38°C.

EXERCISE NO. 20: *Effect of Standing in a Plain and in a Silicone-Coated Glass Vessel on the Rate of Coagulation of Plasma*

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Clotting Mixture: 0.5 ml plasma
0.05 ml. 0.2 M $CaCl_2$

Results:

Duration of Standing (mins)	0	5	15	30	60
Clotting Time (secs)					
Glass	2250	1510	1365	1150	410
Silicone	2280	2170	2270	2310	2270

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Materials: (1) Citrated plasma from rabbit, mouse and man. (2) Thromboplastin prepared by the method described on page 148 using acetone dried (a) human (b) rabbit and (c) mouse brain.

Clotting Mixture. 0.1 ml plasma
0.1 ml thromboplastin
0.1 ml 0.02 M $CaCl_2$

CHAPTER III

MEASUREMENT OF COAGULATION TIME OF BLOOD

1. *Determination of the Clotting Time of Whole Blood*

L. B. JAQUES

Object. The measurement of the rate of clotting of blood after removal from the body

Warning The determination of clotting time is the *simplest*, the *most informative*, and technically the *most difficult* of all common laboratory procedures. Furthermore, continual practice with alert attention to all details is essential. The multiplicity of technics and undeserved censure of the method can be attributed to failure to appreciate this

The test consists of two parts· (a) collection of the blood sample and (b) measurement of the clotting time. Each of these steps must be carefully standardized.

Collection of the Blood Sample

From the standpoint of the sources of error and the significance of the test, this is the more important part of the test The blood sample may be (a) venous blood taken with glass syringes (b) cutaneous blood (c) venous blood taken with silicone (plastics) coated apparatus The selection of methods is determined by the problem If it is necessary to detect a shortening of the clotting time, then a method yielding a long clotting time on normal blood will enhance the possibility of doing this If, however, a quantitative measurement is being sought of blood in a state of extreme hypocoagulability, then a method which will give shorter values in normal blood may be more desirable In addition, selection of the method may be dictated by the *qualitative* nature of the change in blood coagulability under study. In general, the clotting time of cutaneous blood is less sensitive to changes in composition of the clotting system Variable admixture with tissue juices from the puncture site is a drawback and may seriously impair the useful-

Results:

Clotting Mixture	Total Volume ml.	Plasma Conc. %	Thrombopl. Conc mg %	Clotting Time (secs)	
				Normal Plasma	Hemophilic Plasma
0.1 ml. Plasma 0.1 ml. 1-10 dilut T-plastin 0.1 ml. 0 02 M $CaCl_2$	0 3	30	30	26	27
0 4 ml. Plasma 0 1 ml. 1-5 dilut. T-plastin 0 1 ml. 0 08 M $CaCl_2$	0 6	59	30	28	39
1 3 ml. Plasma 0 1 ml. 1-2 dilut. T-plastin 0 1 ml 0 26 M $CaCl_2$	1 5	77	30	32	44
2 8 ml. Plasma 0 1 ml undil. T-plastin 0 1 ml. 0 56 M $CaCl_2$	3 0	83	30	35	48

High concentrations of plasma delay clotting of activated normal plasma, and even more that of hemophilic plasma.

20 gauge steel needle 8 mm test tubes, washed with soap, water, chromate cleaning solution, rinsed well with water and dried. Test-tube rack. Water bath at 25°C. or 37°C. (A pan of water with thermometer and adjusted with tap water just before use is sufficient in ordinary circumstances. A thermos jar is useful for bedside work). Stopwatch.

Procedure. After entering the vein withdraw 3 ml. of blood. Discard 1 ml and transfer the second ml. to a 8 mm. test tube in water bath. Tilt the tube at intervals of fifteen to thirty seconds until clotting occurs. At this time the tube can be turned upside down.

Range: s.d as per cent of mean (log. basis) = 12 per cent when mean is about 8 minutes.

Capillary Tube Method[3, 4]

Materials: Five capillary tubes with lumen of circa 1 mm, length of 5 cm, vaseline, alcohol, automatic lancet, and stopwatch.

Procedure: Clean tip of finger with alcohol and cover with vaseline. Noting the time, make a deep needle puncture so that blood flows *freely* without "milking." Discard first two drops of blood, then allow tubes to draw up the following drops. Fill only ¾ of tube At 30 second intervals tilt tube and notice flow of blood column. When this ceases break off short sections of tube every thirty seconds until a fibrin thread spans the gap between the fragments.

Range: 3–5 minutes.

Silicone Surface Method[1, 5]

Materials 5 ml glass syringe treated with silicone Dri-Film 9977 or 9987 (see page 4) 18 or 20 gauge needle treated with Arquad-2c (see page 5) 8 mm. test tubes treated with silicone, test-tube rack. Water bath at 25 or 38°C (pan of water with thermometer), stopwatch. A thermos jar with water at temperature desired may be used at bedside

Procedure 5 ml. of blood are taken *carefully* using the silicone syringe and needle. The needle is removed and 1 ml of blood discarded Three one ml samples of blood are placed in 3 siliconized test tubes in the water bath and the remainder of the blood discarded If not advisable to take 5 ml, 3½ ml may be used provided the middle portion is used The tube(s) is tilted carefully at intervals until the blood no longer flows The forming of a round surface in contrast to liquid flow provides a definite end-point.

Range: 100–360 minutes Mean—183 minutes (log basis), s d. for replicates is 3 per cent of mean; s d for individuals (6), 7 2 per cent of mean

ness of this source of blood sampling. With venous blood the reactions of the clotting system induced by contact with a glass syringe will mask significant differences in certain components of the clotting system. These may be advantages or disadvantages, depending on the problem.

Measurement of the Clotting Time

There seem to be more methods than investigators in this regard. The essential point is standardization of temperature, container and observation. Clotting is a gradual process. Also it is a general experience that the clotting time is related exponentially with the concentration of any factor. In selecting an end-point, if the final change from unclotted to clotted takes one-half minute for a 4½ minute clotting time, it will take 2½ minutes for a 22½ minute clotting time. Likewise, when variations in thromboplastin in the blood sample, due to variations in trauma of the vein, give a normal clotting time of 4 ± 1 minute, this means a clotting time of 20 minutes will vary between 15 and 25 minutes. Attempts to improve the reading of the end-point cannot alter this situation Mechanical devices are useful in allowing one observer to handle multiple samples. They must not be relied on to improve accuracy or replace continued critical judgment. Both 37°C and room temperature (15, 20 or 25°C.) have been used. The previous remarks on selection of method apply equally to selection of temperature. In all cases, it is essential that sufficient determinations can be done to obtain means with significant standard deviations. For normal values, this must be done frequently It is not unusual to find that over a period of time with continuous practice, the mean normal value obtained by one observer or for one laboratory will change Normal values may be found elsewhere.[1] For the present purpose, of greater significance is the standard deviation (s d.) expressed as a per cent of the mean for a wide range of value of means. To calculate a standard deviation, it is necessary to have a normal frequency distribution curve for the values of clotting time. This can be done by converting the values to corresponding logarithms When the standard deviation is calculated on this basis, it has no absolute meaning. The absolute value of the standard deviation will depend upon the mean value on the logarithmic scale For the Lee and White method, values of the mean range from 5 to 60 minutes.

Three common methods are described. These are sufficient for most purposes. The selection of one of these or a combination of them should be made in the light of the above principles

Lee and White Method[2]

Materials: Glassware, needle, etc must be scrupulously clean. Five ml syringe rinsed with normal saline solution immediately before use; 18 or

2. Cutaneous Coagulation Time

Described by P. J. McKENNA and L. M. TOCANTINS

A. Siliconized Capillary Tube. Method of Lewis and Glueck[1]

Object. The measurement of the rate of clotting of blood in vitro.

Materials Capillary tubes, 150 mm. long, with an outside diameter approximately 0.5 mm, are rinsed with 0 1 N NaOH for 10–15 minut and then rinsed again in three changes of distilled water. The tubes a then immersed in 1:100 aqueous silicone for a few minutes and mixing accomplished by tilting several times. They are rinsed again in three chang of distilled water and dried in an oven at 80 degrees for 3–4 hours Aqueo silicone is prepared by diluting with water.

Procedure: The tip of the finger is cleaned with alcohol and a de needle puncture made so that the blood flows freely. The first few dro of blood are discarded by cotton absorption The blood is drawn by gen suction to about the 75 mm. level of the tube. This suction can be obtain by placing a size 8 French Plastic infant feeding tube over one end of t capillary tube and using mouth suction. The tubes are tilted at 1-mint intervals until the flow of blood ceases. The tubes are then broken at 3 second intervals, and the appearance of a fibrin strand denotes the e point of the test.

Range: Normal subjects—4½ to 7½ minutes

B. Plastic Capillary Tube. Method of Atkinson, Hodges, Atkinson, Du and Frisco[2]

Object· The measurement of the rate of clotting of blood in vitro

Materials· Plastic tubing—8 cm long, 2 8 mm inside diameter alcoh lancet, stopwatch.

Procedure. The tip of the finger is cleaned with alcohol, using the later vascular margin of the distal phalynx, and a deep needle puncture is ma so that blood flows freely The first few drops of blood are discarded l wiping with cotton The blood is drawn by gentle suction to about tw thirds the length of the tubing Beginning at 4 minutes after the punctu the length of plastic tubing is gently tilted every 30 seconds The end po is the cessation of the flow of the column of blood

Range Normal 8–18 minutes

Precautions and Sources of Error. Variations in clotting time obtain by capillary methods will occur if the procedure for collecting the blo is not strictly followed. If a free flow of blood is not effected by fing puncture, then contamination with tissue thromboplastin will invalida the results, thus the greatest source of error lies in the collection of bloo

Sources of Error in Blood Clotting Time Determinations

Collection of the Venous Blood Sample

Most of the variations in values can be traced to this source. The syringe must be a good fit, likewise the needle, and both be scrupulously clean. There must be a good blood flow. The needle must enter the lumen cleanly and must not be caught in the vein wall. It must remain in the lumen. If it catches on endothelium or valve leaflet, the clotting time will be greatly reduced. After centering the needle in the vein, a pause of 2–3 seconds for blood to remove traces of tissue from the end of the needle is advisable. The blood is then drawn as rapidly as possible into the syringe but never so rapidly that the vein is deflated or turbulent flow occurs in the syringe Removal of blood should balance the rate of entry of blood into the vein Conditions such as shock offer a problem in determining clotting times. Very slow removal with a siliconized syringe, is probably best for this. In taking repeated samples, care must be taken not to use the same site. With exposed veins, we have found that 1 cm. is the closest that a second puncture should be.

Determination

Sources of error here are variations in the surface of the tube, i e., degree of cleanliness (see page 8), variations in number of times the tube is tilted and degree of tilting, determination of the end-point. The latter requires experience It should be pointed out that the end-point is quite different to that used in estimating the clotting of plasma. It is equally reproducible.

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Object: The measurement of the rate of clotting of blood in vitro.

Materials: Capillary tubes, 150 mm. long, with an outside diameter of approximately 0.5 mm., are rinsed with 0.1 N NaOH for 10–15 minutes and then rinsed again in three changes of distilled water. The tubes are then immersed in 1:100 aqueous silicone for a few minutes and mixing is accomplished by tilting several times. They are rinsed again in three changes of distilled water and dried in an oven at 80 degrees for 3–4 hours. Aqueous silicone is prepared by diluting with water.

Procedure: The tip of the finger is cleaned with alcohol and a deep needle puncture made so that the blood flows freely. The first few drops of blood are discarded by cotton absorption. The blood is drawn by gentle suction to about the 75 mm. level of the tube. This suction can be obtained by placing a size 8 French Plastic infant feeding tube over one end of the capillary tube and using mouth suction. The tubes are tilted at 1-minute intervals until the flow of blood ceases. The tubes are then broken at 30-second intervals, and the appearance of a fibrin strand denotes the end point of the test.

Range: Normal subjects—4½ to 7½ minutes.

B. Plastic Capillary Tube. Method of Atkinson, Hodges, Atkinson, Dunn and Frisco[2]

Object. The measurement of the rate of clotting of blood in vitro.

Materials: Plastic tubing—8 cm. long, 2.8 mm. inside diameter alcohol, lancet, stopwatch.

Procedure. The tip of the finger is cleaned with alcohol, using the lateral vascular margin of the distal phalynx, and a deep needle puncture is made so that blood flows freely. The first few drops of blood are discarded by wiping with cotton. The blood is drawn by gentle suction to about two-thirds the length of the tubing. Beginning at 4 minutes after the puncture, the length of plastic tubing is gently tilted every 30 seconds. The end point is the cessation of the flow of the column of blood.

Range. Normal 8–18 minutes.

Precautions and Sources of Error. Variations in clotting time obtained by capillary methods will occur if the procedure for collecting the blood is not strictly followed. If a free flow of blood is not effected by finger puncture, then contamination with tissue thromboplastin will invalidate the results, thus the greatest source of error lies in the collection of blood

The clotting time of cutaneous blood is less sensitive to changes in composition of the intrinsic coagulation system than venous blood. In general, capillary coagulation times should be used only when it is impractical to obtain adequate venous samples. The results obtained from cutaneous blood should be interpreted with caution. If prolonged times are obtained by this method, these values are far more significant than if normal values are obtained. If the shortcomings of the method are recognized, this method will have definite but limited application, particularly in children with coagulation defects.

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[1] Lewis, R. C and Glueck, H.. A micromethod for determining clotting time, using capillary blood and siliconized tubes. J Lab & Clin Med. 32 299-303, 1948.
[2] Atkinson, J. Hodges, J. Atkinson, G R, Dunn, D. and Frisco, L: Coagulation times of blood collected from cutaneous punctures as determined in plastic capillary tubing Am J Clin. Path 35 284-287, 1961.

3. *Thrombelastography. A Method for Continuous Recording of Fibrin Formation and Fibrinolysis*

K. N. von KAULLA and E. von KAULLA

Object of Method The principal object is to obtain a graphic representation of the entire course of the clotting process rather than information on one point only of the clotting curve as the great majority of clotting measurements do. This object is achieved by continuous recording of the speed and kinetics from onset to completion of fibrin formation in blood or plasma which clots Further objects of the method are to measure continuously the speed and kinetics of fibrinolytic clot dissolution and to produce automatically permanent records of clot formation and clot dissolution

Principle of Test The rate of growth of fibrin strands in blood or plasma is continuously converted into oscillations of a light beam The oscillations to be seen on a calibrated transparent scale are simultaneously recorded on a film The conversion is effected in such a way that the clot firmness at any moment during and after clot formation is reflected by the amplitude of the oscillations of the beam of light, which also produces on the film a sigmoid clotting curve together with its mirror-like image along the horizontal axis (see fig. 1)

General Remarks: The tracings produced by the thrombelastograph are known as thrombelastograms or coagulograms. Thrombelastograms of whole blood indicate variations of the coagulability of the blood much better than other methods, for instance, the Lee-White time. The sensitivity of the method is further increased with the use of plasma, in particular citrated plasma (for exception see section of precautions and sources of errors). The thrombelastograph has been shown to be of great value in screening for undetected clotting deficiencies, for detection of hypercoagulability and non-thrombocytopenic platelet abnormalities, in typing for clotting factor deficiencies (correction of the thrombelastogram upon addition of the deficient factor), for obtaining additional information on the effectiveness of indirect anticoagulants, and for measurements of evolution of clot firmness and of the kinetics of clot lysis. In our hands, the thrombelastograph is of particular interest for guidance and control of substitution therapy during major surgical procedures in hemophiliacs and for surveillance of the clotting process in connection with cardiac surgery.

Apparatus: Thrombelastograph* according to Hartert, with double set of cups and cylinders, manufactured by Hellige A. G., Freiburg, Breisgau, West Germany. The machine has three identical measuring units, which allow simultaneous study of three specimens. The following are the most essential technical features: A stainless steel cup, polished inside, 12 mm. high, 8 mm. i.d., is shaped to fit tightly in a round socket which oscillates the cup in a total angle of 4°45". The rotary movement to either side lasts 3.5 seconds and is followed by a resting period of 1 second. The total cycle requires 9 seconds. The three sockets to receive the cup are built into a 37°C. thermostat. The specimen to produce the thrombelastogram is poured into the cup (usual volume, 0.36 ml.) which is then placed in the socket. Centered above each cup, suspended on a 0.1 mm. stainless steel torsion wire is a polished stainless steel cylinder. The torsion wire is fitted with dampening vans which dip into mineral oil to protect the sensitive torsion system from vibration. A small mirror is attached to the upper end of the torsion wire. By releasing a blocking mechanism which protects the measuring unit when the machine is not in use, the cylinder is lowered into the cup in such a manner that it hangs free on the torsion wire at a uniform distance of 1 mm. from the inner wall of the cup and is covered by the test solution. The clot then forms in the space between the wall of the cup and the cylinder. Cup wall and cylinder are progressively more firmly connected to each other by fibrin strands as the fibrin formation progresses, consequently as the cylinder follows the movements of the cup more closely,

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* Distributed in the United States and Canada by Haemoscope Corp. Albertson, L. I., N. Y.

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References

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Thrombelastograms with whole blood.

(1) The cylinders are tightly inserted into the hole below the dampening vans. When they slip into position, a clear "click" is heard. Each pair of cylinders and cups have matching letters and should always be used together in the same measuring unit. The units are numbered

(2) The thrombelastograph is switched on and the thermostat allowed to warm up to 37° C. About 10 minutes are required. This time should be checked for each individual machine. Moreover, the temperature should be checked from time to time with a good thermocouple while the cups are filled with water and the cylinders are lowered into the cups

(3) A clean venepuncture with a minimum of stasis is performed, a few milligrams of blood withdrawn, the syringe removed, and the blood allowed to drip rapidly from the needle secured in the vein into the cup until the cup is about two-thirds full.

(4) The cup is next immediately placed into the socket, the cylinder lowered by turning the knob on top of the measuring unit until there is a clear stop. At this time a colored line of light appears on the scale. The light line is shifted, if necessary, to its assigned place on the scale (identified by colored numbers corresponding to the units) by turning the knob in front of the measuring units The time from drawing blood to lowering the cylinder should be as short as possible and should not exceed 2 minutes

(5) The film is started at the same time the cylinder is lowered (or earlier, but not later) by pulling the knob on top of the housing of the photokymograph.

(6) In order to avoid drying out, the specimens are carefully covered with a few drops of mineral oil Use a syringe with a hypodermic needle.

(7) The thrombelastograph can be run for any length of time. The use of a timer permits automatic shut-off.

Thrombelastograms with plasma

Steps 1 and 2 are the same as for blood Step 3 is modified as follows. After a few ml of blood have been withdrawn, the syringe is taken off and a second 10 ml. syringe, loaded with 2 ml 3 8 per cent sodium citrate, is used to withdraw blood to the 10 ml. mark without bubbles. [illegible]

n International Clinical Centrifuge Model CL. Thereafter the plasma is recalcified by putting 0 33 ml plasma into the cup and pipetting 0 03 ml. 0.5 M $CaCl_2$ into it, whereupon the cup is placed immediately into the machine The next steps 4 to 7 are the same as described for the whole blood

this connection—the fibrin strands—grow stronger. The small clearance of the cylinder and the limited excursion of the cup reduce to a minimum the mechanical distortion of the clot formed in the cup during the run. With the cylinder in the lowered position, a beam of light filtered through a narrow slit is projected onto the mirror. The light reflected by the mirror is split into two beams, one is thrown onto the film; the other, after having passed through a colored lens (with different colors for each of the three measuring units), is projected onto the transparent graduated scale where it can be observed. Non-clotted blood or plasma does not bind the cylinder to the cup; therefore the cylinder is not moved and the light beam is seen motionless on the scale, appearing as a straight line on the developed film. When sufficient fibrin is formed to bind the cup strongly enough to the cylinder so that the very small resistance to torsion of the torsion wire can be overcome, cylinder and mirror start to oscillate, and the light beam begins to exhibit excursions, the amplitude of which grows wider as the strength of the fibrin connection between cup and cylinder increases. It should be noted that the light beam does not start to swing when the very first fibrin strand is formed but only when the fibrin formed is strong enough to twist the torsion wire. The required amount of firm fibrin to do this is small: thrombelastograms of recalcified citrated human plasma diluted 1:10 are readily obtained

The light beam, not moving for 1 second at the end of each excursion before it swings in the opposite direction, frames the area covered by the oscillating light with a black line on the film. In most machines the film moves 2 mm a minute In the thrombelastograph used in this laboratory, the film moves 1 mm /minute which is considered more convenient, particularly for recording delayed coagulation or fibrinolysis. A prototype of a "2 mm" thrombelastogram is shown in figure 1

Reagents 3 8 per cent citrate U.S.P., 0.5 M $CaCl_2$; mineral oil U.S P.; barium sulfate X-ray quality, disposable hypodermic needles 20 gauge, 1½ inch long, B-D #1000, 10 ml. syringes with straight tip, B-D multifit (preferably siliconized), 12 ml. heavy-wall centrifuge tubes Corning # 8120; timer, Westinghouse TC-81; adsorbent cotton. Film, Agfa 15 m, 100 mm., perforated, emulsion 1., Agfa Aktiengesellschaft, Leverkusen, Germany. Reagents for developing and fixing film (solutions prepared for X-ray films are convenient)

Steps for Producing Thrombelastograms: The thrombelastogram is obtained either from whole blood or recalcified plasma We do not recommend oxalated plasma which is less sensitive for minor clotting deviations than citrated plasma Thrombin-clotted specimens produce abnormal and narrow thrombelastograms.

Thrombelastograms with whole blood.

(1) The cylinders are tightly inserted into the hole below the dampening vans. When they slip into position, a clear "click" is heard. Each pair of cylinders and cups have matching letters and should always be used together in the same measuring unit. The units are numbered.

(2) The thrombelastograph is switched on and the thermostat allowed to warm up to 37° C. About 10 minutes are required. This time should be checked for each individual machine. Moreover, the temperature should be checked from time to time with a good thermocouple while the cups are filled with water and the cylinders are lowered into the cups.

(3) A clean venepuncture with a minimum of stasis is performed, a few milligrams of blood withdrawn, the syringe removed, and the blood allowed to drip rapidly from the needle secured in the vein into the cup until the cup is about two-thirds full

(4) The cup is next immediately placed into the socket, the cylinder lowered by turning the knob on top of the measuring unit until there is a clear stop At this time a colored line of light appears on the scale. The light line is shifted, if necessary, to its assigned place on the scale (identified by colored numbers corresponding to the units) by turning the knob in front of the measuring units. The time from drawing blood to lowering the cylinder should be as short as possible and should not exceed 2 minutes.

(5) The film is started at the same time the cylinder is lowered (or earlier, but not later) by pulling the knob on top of the housing of the photokymograph

(6) In order to avoid drying out, the specimens are carefully covered with a few drops of mineral oil Use a syringe with a hypodermic needle

(7) The thrombelastograph can be run for any length of time The use of a timer permits automatic shut-off

Thrombelastograms with plasma

Steps 1 and 2 are the same as for blood Step 3 is modified as follows: After a few ml of blood have been withdrawn, the syringe is taken off and a second 10 ml syringe, loaded with 2 ml 3 8 per cent sodium citrate, is used to withdraw blood to the 10 ml mark without bubbles Syringe and needle are taken off, the needle is removed and the content of the syringe gently mixed by pulling the barrel back and tilting the syringe. The citrated plasma is centrifuged for 5 minutes at full speed (1470 g) in an International Clinical Centrifuge Model CL Thereafter the plasma is recalcified by putting 0 33 ml plasma into the cup and pipetting 0.03 ml 0 5 M $CaCl_2$ into it, whereupon the cup is placed immediately into the machine The next steps 4 to 7 are the same as described for the whole blood

This procedure with plasma is our personal method designed for high sensitivity and to avoid significant dilution of the specimen.[3] Other investigators use different relations of the volume of $CaCl_2$ to the recalcifying agent, for instance, 0.26 ml. oxalated plasma recalcified with 0.1 ml. 1.29 per cent $CaCl_2$ added with an insulin syringe fitted with a curved hypodermic needle.[1] In this case, the cup with plasma is placed into the socket and temperature equilibration is allowed prior to recalcification The temperature equilibrium is reached in about 5 minutes. We did not observe significant differences in the thrombelastogram whether or not temperature equilibrium has been reached before recalcification.

Film. The photographic recordings can be taken out and developed during the run. However, it is more convenient to do so after one or several runs. The developed and fixed recordings are to be exhaustively rinsed before drying.

Cleaning of cylinders, sockets, and cups. Careful cleaning is crucial. After completion of the run, the cylinders are turned upwards until the knob on top of the measuring unit cannot be turned further and cylinders and cups are removed and cleaned as follows: (1) under running hot tap-water remove oil, blood, plasma and clot traces by means of cotton swabs; (2) wipe and rub thoroughly with cotton swabs (wrapped around applicator sticks) which have been dipped into a shaken suspension of $BaSO_4$ in water (1 gm./20ml.); (3) rinse under running hot tap-water, (4) remove the last traces of $BaSO_4$ with cotton swabs; (5) rinse with hot tap-water followed by distilled water; (6) wipe dry with a new set of cotton swabs for each cup and cylinder; (7) store protected from dust. After cleaning, the cylinder cannot be touched any more with the fingers. If it happens, wipe again with new cotton. The socket is also to be cleaned after each run, because small amounts of dirt or liquid prevent the cup from fitting properly.

Expression of results: The thrombelastogram is conveniently divided into several sections, as shown in figure 1 Below are indicated the normal values together with their section designations for whole blood with the use of 2 mm /min. film speed. The values for recalcified plasma are similar but not identical and vary with the type of decalcifying agent and the recalcification technic used

E represents the time elapsed from the drawing of blood until the cylinder is submersed in it (Plasma time from recalcification to submersion) On the thrombelastogram, r corresponds to the time from beginning of E until the thrombelastogram has reached the width of 1 mm This requires about 8 to 9 minutes and it is thought to represent the thromboplastin formation time K designates the interval from the end of r until the thrombelastogram is 20 mm wide. K is thought to be equivalent to the thrombin formation time; r plus K is considered to be roughly e

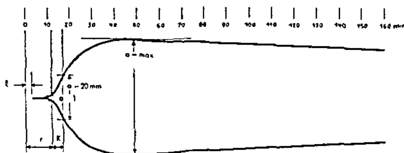


FIG 1.—The sections of the thrombelastogram (for details, see text).

to the clotting time observed in the test tube. Actually the clot formation continues until the maximum width of the thrombelastogram, called max or ma (54 to 62 mm) is obtained After max is reached, the thrombelastogram becomes somewhat smaller in most instances, indicating, according to some investigators, retraction.

The average error (triplicates) for r is about 2 per cent, for r + K 3 per cent, and for max 1 per cent.

Because always the same force is required to inflict the same amount of torsion on the torsion wire, the torsion wire itself serves as a reference standard.

Interpretation of Results Prolonged r is indicative of clotting deficiency, short r with short K or short K with normal r indicates hypercoagulability Moderate deficiencies of components of the prothrombin complex do not necessarily prolong r Reduced max is suggestive of platelet deficiencies (in number of function), particularly if accompanied by a blackish fuzzy shadow along the middle axis A narrow thrombelastogram is also produced by pronounced hypofibrinogenemia A progressive reduction of max after its peak indicates progressive lysis of the clot [2] Sudden interruptions of the smooth outlines of the thrombelastogram reflect structural aberrations of the clot, described to occur with platelet abnormalities or in the presence of pathologic leukocytes For this particular phenomenon, whole blood is more sensitive than plasma; all other potential variations of the thrombelastogram are easier to demonstrate with recalcified citrated plasma For clotting analysis of unknown abnormalities, it is recommended that thrombelastograms be run with both whole blood and with recalcified citrated plasma whenever possible

Precautions and Sources of Error Main sources of error are dirt, traces of old blood, plasma, detergents, and fingerprints on the cylinder Secondly

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CHAPTER IV

MEASUREMENT OF THE RATE AND EXTENT OF CLOT RETRACTION

L. M. TOCANTINS

Object A quantitative estimation of how much and how fast a clot retracts after it has been formed.

Principle: The method is essentially like that of Adreassen. Blood is allowed to clot in a paraffin coated or siliconized vessel in the center of which is an uncoated glass rod. The blood adheres to the glass but not to the paraffin, thereby making it possible to invert the vessel and measure the expressed serum, while the clot remains adherent to the glass

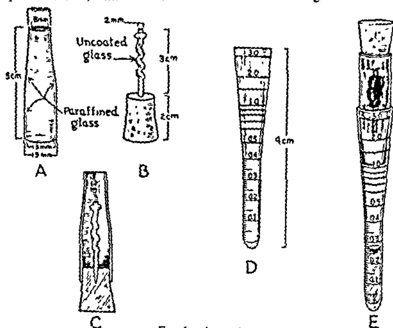


FIG 1 —Apparatus.

are faulty specimens drawn with tissue thromboplastin contamination, use of a specimen which has started to clot (not necessarily visible) before the cylinder is submerged in it, and, most important. plasma specimens which have been "stored." Blood must be processed for plasma and the run started as soon as the blood has been drawn (exception. studies on the effect of storage on the thrombelastogram). These effects are variable but considerable and affect all parts of the thrombelastogram Other sources of error are incomplete lowering of the cylinder, and rapid sedimentation of the erythrocytes before the blood is clotted. In both instances, only one-half of the thrombelastogram might be produced and the light on the scale is shorter than normal. Omission of the oil cover allows the specimens to dry out and produces abnormal wide thrombelastograms with irregular outlines. The oil on top of the specimens introduces some minor alterations. We have designed special teflon screw caps to overcome this difficulty. Their use is, however, rarely necessary. Unequal filling with mineral oil of the receptacula of the dampening vans might affect the comparability of the values obtained with the individual measuring units Removal of platelets by too strong centrifugation introduces marked errors. Scratches on the polished surfaces of cups and cylinders make them useless. Vibration of the machine during the run (a door thrown shut) can disrupt the fibrin strands and alter the trend of the tracings

The temperature of the content of the cup with the lowered cylinder in it may vary several degrees during an automatic switching "on" hand "off" cycle of the thermostat Information (and, if necessary, correction) on this point for the individual machine in use is important.

The thrombelastograph should be protected from vibration similar to an analytical balance

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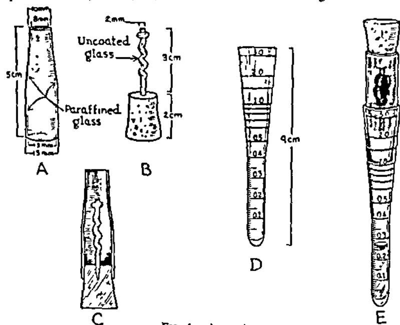


Fig. 1 —Apparatus

Apparatus: (fig. 1) A tapered glass tube (A) closed by a cork stopper on the bottom to which a gnarled glass rod has been attached (B) the inside surface of the tube and the cork bottom are coated with paraffin (M P. 50°C.) but the glass rod is left bare. When these are assembled they appear as in (C). A graduated tube (D) like that used for the Addis urine count technic.

Steps in the Procedure: Collect 2 ml. of venous blood and place exactly 1.0 ml in the assembled tube which is then stood up in the incubator at 38°C. The rest of the blood is used to determine the per cent packed cell volume (hematocrit). Determine the clotting time of the blood in the tube by tilting it at intervals. When clotting is complete, allow one hour to elapse, then invert the small tube and insert it into the graduated tube as shown in (E) (fig. 1). Read the volume of the serum collected at the graduated stem of the tube. Reexamine in another hour Then remove the gradu-

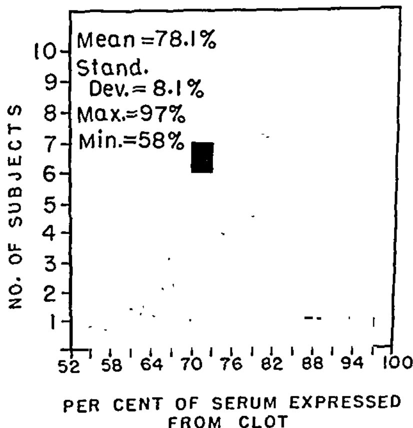


FIG 2.—Retraction (2 hours) of clots from 1 cc of venous blood in a paraffin tube, 13 mm i d at 37°C (59 adults)

ated tube and centrifuge for 15 minutes at 2000 rpm Read the level of packed cells and the expressed serum.

Calculation: Results are expressed in terms of the percentage amount of serum expressed from the clot at the end of 2 hours taking into consideration the amount of potential serum present in the clot (calculated from the hematocrit determination) *Example* 1.0 ml. of blood with a percent cell volume of 46 expressed 0.4 ml. of serum at the end of 2 hours.

$$\text{Percent serum expressed} = \frac{0.4 \times 100}{0.54} = 74\%$$

0.54 ml. is the total amount of serum potentially present in 1.0 ml. of the specimen of blood.

Range of Values: Mean of 59 determinations in 59 adult men and women: 78.1 per cent (Stand Dev. 8.1 per cent); range 97–58 per cent. The frequency distribution is shown in figure 2.

Precautions and Sources of Error: The glass rod must be left uncoated or the clot will not adhere to it. When the rate of clotting is unusually slow or imperfect (as in hemophilic blood) the method is unreliable, since partial coagulation renders the clot nonadherent and easily displaced. The "serum" expressed is in reality only partly serum.

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CHAPTER V

BLOOD PLATELETS

1. Counting Platelets in the Blood

L. M. TOCANTINS

Most technical methods for counting platelets utilize either cutaneous or venous blood. The choice of methods obviously must depend to a certain extent on the experience of the one who is to use the method For *inexperienced* workers, the order of choice should be. (1) indirect platelet count on cutaneous blood with an isotonic solution containing a stain, a fixative and an anticoagulant; (2) indirect count on venous blood; (3) direct platelet count on cutaneous blood; (4) direct platelet count on venous blood The ratio of erythrocytes to platelets should be established from at least 1,000 erythrocytes. The choice of methods is further conditioned somewhat by the type of blood to be examined With leukemic and thrombocytopenic blood, counting on wet specimens should be supplemented by a method in which the dry stained smear is used, such as Fonio's, as a further check on the number of platelets or as a means of avoiding the inclusion of any hyaline, platelet-like bodies in the total count. Venous blood is preferable to cutaneous blood. The trauma involved in an incision of the skin is greater than that in a venous puncture with a small needle (from 26 to 27 gauge). When one considers the role played by the platelets in repairing the effects of trauma, it is logical to anticipate that the trauma involved in an incision of the skin will affect the number of the platelets. Comparative determinations of the platelets in vessels with and without trauma yield great differences, and this fact renders serial counts from the same location within short periods of time of questionable value. Appreciable numbers of platelets are lost in the lips of the wound before the blood reaches the sur- [illegible] Preiss,[1] the blood passes [illegible] of the needle, where it [illegible] ulant and (preferably) fixed, all surfaces of contact having previously been wetted by the solution Counts done on venous blood are more representative of the actual conditions in the circulation, for a larger amount of blood is usually collected for examination than when a cutaneous puncture is used. It is also possible to

repeat the count and make observations on the other morphologic constituents of the blood.

For greater accuracy, in experienced hands, direct methods are preferable to indirect ones. Most of the objections usually raised against the use of the direct method apply only if it is employed by one who is inexperienced with the manipulative procedures involved Even on cutaneous blood, if there is free bleeding from the wound, rapid coordination of movements, use of scrupulously clean glassware and solutions, duplicate determinations and uniform shaking, this method is useful.

Some of the objections to the indirect method of counting are: The mixture of blood and diluting solution is seldom, if ever, uniform and not the same each time. The ratio of erythrocytes to platelets in a given sample of blood determined on the counting chamber often varies widely from that determined in wet smears. Platelets and erythrocytes are not distributed evenly through the preparation since no provision is made for shaking before counting It is obvious that the greater the number of erythrocytes and platelets counted, the more accurate will be the ratio established between these two elements, if a counting chamber is used to establish this ratio, the total number of erythrocytes counted will vary, depending on the dilution employed The method has defects intrinsic in any determination done indirectly, that is, in relation to another equally changeable element. The greatest sources of error, however, is in the assumption that platelets and erythrocytes keep an even proportion in numbers toward each other between the two main steps of the method The markedly different physical properties (adhesiveness, specific gravity, size and others) of platelets and erythrocytes lead to continuous changes in this ratio Finally, the proportion of platelets to erythrocytes varies at the same time in different portions of the circulation, and this variation is even more marked in capillaries and venules

With the Thomsen[2] method and its modifications, the platelets are counted in the plasma, free from other cells This is advantageous in some respects; it decreases the possibility of confusion with the products of the disintegration of erythrocytes besides having all the other advantages of methods in which venous blood is utilized Although the Thomsen method at first seems a direct method of counting platelets in whole blood, it actually is an indirect method, for one must always have a supplementary determination of the volume of packed cells or of the hemoglobin to calculate the absolute number of the platelets per cubic millimeter of blood This multiplicity of determinations may contribute to increase the error, but, unlike the indirect method in which use is made of the erythrocyte-platelet ratio, the determinations are all done in the same sample of blood There is also some question as to the stability and uniformity of the suspension of

platelets in plasma, during the time required for sedimentation of the erythrocytes.

As anticoagulants, sodium citrate, sodium oxalate, sodium metaphosphate, sodium or magnesium sulfate, heparin, peptone and gelatin have been used in diluting solutions. Of these, sodium citrate seems to be the best. Oxalates often form crystals and precipitates with the plasma. Sodium metaphosphate should be in higher concentrations (from 5 to 10 per cent) than those generally used (2 per cent). Both peptone and gelatin, like sucrose and dextrose, encourage the growth of bacteria. None of these anticoagulants prevents morphologic changes in the platelets without the addition of a fixing reagent The preservation of the morphologic character of the platelet for total and differential counting may be obtained only by the dual process of simultaneous prevention of coagulation of the blood and rapid fixation. Good fixation depends not so much on a high concentration of the fixative, as on the promptness with which even a weak fixative comes in contact with the platelets after these have left the vessels As fixing reagents, mercury bichloride, 1 and 2 per cent osmic acid, solution of formaldehyde U.S.P, methyl and ethyl alcohol and potassium bichromate have been used. Of these, solution of formaldehyde U S.P. in a concentration of about 0 1 per cent seems the best. Diluting fluids containing this solution of formaldehyde in percentages of 2 and 2.5 per cent often hemolyze erythrocytes and form precipitates with the plasma. It is important to use neutral solution of formaldehyde in the preparation of solutions, for if enough formic acid is present it will hemolyze the red blood cells. When a solution containing formaldehyde has stood for a time, some of the formaldehyde is oxidized and formic acid is formed When this acid reaches a concentration of from 0 05 to 0 075 per cent it decolorizes the erythrocytes even in isotonic salt solution, although it does not affect platelets or leukocytes The hemolysis that is occasionally encountered when one is using the Rees-Ecker solution is chiefly due to this. When a diluting fluid containing formaldehyde begins to hemolyze the red blood cells it should be discarded, and the glassware that has been in contact with it should be thoroughly cleaned Methyl and ethyl alcohol are poor fixatives for platelets in wet preparations, for they precipitate the plasma proteins and cause secondary changes in the platelets Mercury bichloride in a concentration such as that in Hayem solution B (0.25 per cent) seems a good fixative In higher concentration it often produces protein precipitates Osmic acid, while an excellent fixative for dry smears, is expensive and often produces clumping of erythrocytes in wet preparations Approximately half of the diluting [illegible] do not include a fixative

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violet, methyl green, neutral red, methylene blue and eosin have been used. Brilliant cresyl blue in a concentration of from 0 05 to 0 025 Gm per hundred cubic centimeters seems the best. Methyl violet is satisfactory, while the others, like methyl green, stain the platelets poorly or not at all. For counting on dry smears, the Giemsa or Wright stain demonstrates the platelets best Stains aid the reading of the meniscus in pipets and syringes but increase the chances of producing artefacts and the labor of cleaning glassware.

The use of diluting solutions that hemolyze the red corpuscles is generally inadvisable The erythrocyte count which should accompany every platelet count is made impossible, many artefacts are created, and it is difficult to be sure that the platelets themselves are not sometimes affected. In experienced hands, however, these solutions may be used advantageously. The hemolyzing agents employed have been potassium cyanide and urea

Diluting solutions resembling the plasma in composition, such as Tyrode and Ringer solution, offer no especial advantage in the counting and morphologic study of intact platelets Their similarity to plasma in composition may be a handicap in this study, for there are few fluids in which platelets disintegrate more rapidly than in plasma collected without precautions to prevent its coagulation Adding a fixative, such as mercury bichloride, to Tyrode solution remedies the disadvantages of this solution partially, but such an addition obviously alters its delicate chemical balance, thus defeating the original purpose in using it and making its use no more advantageous than that of other far simpler solutions Both Tyrode and Ringer solution encourage the growth of bacteria, neither keeps well, and the multiplicity of chemical substances in each is a constant source of artefacts. Methods in which Tyrode diluting fluid is utilized invariably yield high platelet counts on cutaneous blood The platelets observed in excess over the usually accepted normal number are, however, of the "micro" type, and it has been demonstrated that most of these forms are artefacts, formed either by contact of the blood with tissue juices in the presence of Tyrode solution or by precipitation of the calcium chloride of the solution when this is in contact with the alkali of the glass

Pipettes and syringes of special shape and composition have been employed; although useful in the hands of their originators, they offer little advantage over the standard types available Counting chambers such as that of Helber (depth, 0 02 mm ; cover glass thickness, 0 1 mm), the Petroff-Hausser counting chamber for bacteria (depth, 0 02 mm , cover glass thickness, 0 18 mm) are useful but do not seem strictly necessary The standard Burker, Thoma-Zeiss and Levy-Hausser counting chambers

(depth, 0.1 mm.) and the Thoma diluting pipettes, if properly calibrated and certified, seem entirely adequate.

A magnification of from ×400 to ×600 with dry objectives seems best. Higher magnifications may be obtained with chambers less than 0.1 mm. in depth and with use of thin cover glasses. Oil immersion objectives often disturb the cubic content of the chamber, and the use of low power objectives is not advisable. While identifying platelets, the observer should have the fingers of one hand on the fine adjustment to obtain the critical focusing that reveals the characteristic highly refractile, silver chip appearance of the platelet. Only forms from 1 to 3 microns or longer in size, rod or comma-like if seen sidewards, and thin, translucent and disklike if flat on the floor of the counting chamber, should be counted. Granules 0 8 micron in diameter or smaller, jerkily moving about more or less actively, globules of oil, irregularly shaped debris floating on the upper layers of the fluid, strings of cocci and other minute objects may be distinguished from platelets after a little practice. The error of counting too few platelets may be equaled only by the error of counting every particle in the field as a platelet.

Many of the suggested precautions are often useful under certain conditions Platelets may be counted, however, just as easily as erythrocytes if coagulation of the blood is effectively prevented and the platelets are fixed as the blood is withdrawn, without contact with the tissues or any wettable surfaces. It is the initial stage of alteration in the platelet that renders it so strongly adhesive and agglutinable Rapidly fixed platelets preserve their size and shape and adhere very little to glass surfaces or to each other. Therefore, a diluting fluid for total and differential platelet counting should: (1) effectively prevent coagulation of the blood and changes in the platelets; (2) fix the platelets as the blood is being removed from the vessels; (3) be isotonic with the blood and cause no precipitates with the plasma or the glassware, and (4) be of simple composition and of easy preservation Of the fluids available for platelet counting, the Rees-Ecker solution (of which the solution to be described is only a slight modification) seems to have all the necessary ingredients in the best combination A stain is necessary only until one has gained experience in recognizing platelets. The following solution has been found to fulfill the requirements sodium citrate, 3 8 Gm.; solution of formaldehyde (neutral, 40 per cent), 0.2 ml , distilled water, 100 ml If a stain is desired, add 0.05 Gm of brilliant cresyl blue. Keep in a well stoppered bottle in an icebox, filter each time just before using, per- [illegible] cteria, molds [illegible] The use of

Method for Counting Platelets in Venous Blood

Apparatus: a syringe of the type used in making the tuberculin tests, having a capacity of 0 5 ml. and graduated in tenths or five hundredths of a milliliter; vials of 2 ml. capacity, containing a glass bead and having glass stoppers ground to fit; an automatic shaker; pipette and counting chambers certified by the United States Bureau of Standards. The solution is kept at from 2° to 4°C. after being filtered. It is placed in small test tubes and sterilized in the autoclave along with tubes containing distilled water, loss in fluid in the solution during sterilization being made up with the distilled water. The needles, syringes and vials are sterilized by dry heat. Counting chambers and cover slips are boiled once weekly for two hours in distilled water containing a small amount of sodium bicarbonate; pipettes are cleaned by the usual changes of water, ether and alcohol.

Steps in the Procedure: Aspirate 0 4 ml of the diluting fluid into the syringe and dry the tip with a sterile gauze stopper. Adjust a sterile No 27 gauge needle to the tip snugly, to prevent any ingress of air Then carefully push the solution up the shaft of the needle until it just appears at the lumen For needles of the size specified, 0 03 ml. of solution is usually needed to fill the shaft of the needle. This, therefore, will displace the piston downward. Transfer to the sterile vial enough of the solution to bring the piston down to coincide with one of the rulings on the barrel. Enter the vein after distending it slightly by application of a little pressure above the site of puncture. Once the needle is in the vein, remove the pressure, and allow approximately five seconds to elapse before withdrawing blood Slowly withdraw exactly 0.1 ml of blood into the syringe. Then place the tip of the needle under the level of the solution in the vial, and empty the contents of the syringe into it, aspirating and emptying the solution and blood slowly, without bubbling, three or four times to insure thorough mixing Stopper the vial and place it in an automatic shaker, where it is to be rhythmically agitated for at least five minutes From the shaken mixture make a 1 100 dilution, using a diluting pipette and the same diluting fluid Submit this dilution also to shaking, then place a drop of it in two counting chambers, and count the erythrocytes in 160 of the small squares of each chamber, multiply the average of the two counts by 12,500 Allow the preparation to stand in a moist chamber for at least twenty minutes before counting the platelets. Count all platelets in 400 small squares (or the entire finely ruled area of each chamber) and multiply the average of the two counts by 5,000

In man, the basilic veins in the forearm are used for venous platelet counts; in dogs, the leg, arm or ear veins, and in rabbits, the ear veins For platelet counts on arterial blood in man, the radial artery is the most accessible; the brachial artery, at the elbow, may also be used, in dogs the

femoral artery, along the thigh, is the most accessible. Counts done at the same time on blood from various channels are done preferably in this order. (1) venous blood; (2) arterial blood; (3) cutaneous blood. Whenever the syringe is used for making dilutions, it is essential for the operator to place himself and so arrange the arm of the subject that the syringe is held horizontally. This insures easy reading of the marks on the syringe without making errors because of parallax, and insures detection of the moment when the artery is entered, for the pressure in the vessel will overcome the column of solution in the syringe if the latter is held horizontally. The syringe should be so turned that the graduation lines are on each side of, and not over, the piston. Measurements are made by adjusting the end of the piston along the same line as graduation markings on the side, thus preventing parallactic errors, which are unavoidable when the end of the piston is adjusted under the ruling.

If the platelets are above 1,000,000 per cubic millimeter, dilutions higher than 1.5 are used to reduce the chances of agglutination The contention that high dilutions of blood yield correspondingly higher counts because of fragmentation of platelets applies only when no fixatives are used. If there is reason to expect a low platelet count, the blood is diluted 1 2 in the syringe instead of 1:5, and special precautions should be taken with the diluting fluids. If the platelets in the chamber are few, the presence of any extraneous matter in the diluting solution will often cause the most experienced technician to include them in the count if they resemble platelets The most scrupulous cleaning of the glassware and filtering of the solutions will leave a very small amount of particles in suspension When the platelet count is normal, these particles usually do not greatly alter the result, since in any solution they should not be present in greater concentration than 5 particles per tenth of a cubic millimeter of the solution When few or no platelets are present, however, they may look enough like platelets to cause the observer to count them as such, thereby greatly exaggerating the platelet count If the diluting solution includes a dye like brilliant cresyl blue, this difficulty is increased For this reason, with every series of platelet counts, a blank count on the filtered solutions should always be done and the result subtracted from the platelet count if the solution contains only 5 or less platelet-like bodies per tenth of a cubic millimeter This practice not only increases the accuracy of the count but gives also a frequent check-up on the solutions The total number of platelets is calculated by subtracting the number of platelets counted in the blood dilution preparation (a) from the number of platelet-like bodies per tenth of a cubic millimeter in the diluting solution (b) according to this formula.

$$\frac{(a - b) \times \text{dilution} \times 4{,}000}{400} = \text{number of platelets per cu. mm of blood}$$

In thrombocytopenic bloods, platelets are further searched for in a smear of blood made from the mixture and stained with Wright or Jenner-Giemsa stain; after the erythrocytes have settled in the vial, a loopful of the supernatant plasma is examined for the presence of platelets as a hanging drop. Occasionally(when the count is very low) the drop of plasma may be introduced without further dilution into the counting chamber, the platelets in 400 small squares counted, and the total number of platelets per cubic millimeter of plasma obtained by multiplying the total number counted by 10.

Values found Mean of 40 determinations on 40 normal adult men· 310,000 per cu mm. (max.: 690,000. min.: 150,000).

Method for Counting Platelets in Cutaneous Blood

Rees-Ecker Direct Method. Aspirate the following freshly filtered solution up to point 0.5 of a pipette for counting erythrocytes: sodium citrate 3 8 Gm ; 40 per cent formaldehyde solution 0.2 ml ; brilliant cresyl blue 0.1 Gm. and distilled water 100 ml. Make a puncture 3—4 mm. deep in the finger and from the freely bleeding wound, aspirate enough blood to push column up to point 1. Fill the remainder of the pipette with the solution up to 101. Shake, place the 4th or 5th drop in the counting chamber (Levy-Hausser), wait for 15–20 minutes for settling and then count the platelets in the entire finely ruled area (400 squares). Multiply the total by 2,000 to obtain the number of platelets per cu mm. of blood

Values Found· Mean platelet count in 40 young adults 250,000 Range *(2x stand dev.) 243,000–257,000*

Indirect Method on Dried Stained Blood Smears (Modified Fonio)· Place a large drop of 14 per cent magnesium sulfate solution over the skin of the finger, which must be clean and dry. Puncture the skin through the liquid, collect a drop of the blood-liquid mixture with a paraffin-coated glass rod, place it on a clean slide and make a smear Fix the smear in absolute methyl alcohol (two minutes) and stain for from three to five minutes with Wright's stain (10 drops of stock Wright's solution added to 10 drops of Wright buffer) Wash with distilled water, dry and examine with the oil immersion lens Establish the ratio of platelets to erythrocytes from at least 1,000 erythrocytes Calculate the number of platelets from this ratio and an erythrocyte count done at the same time

Values found Mean platelet count in 30 male adults· 234,000. Range Max 350,000 Min 130,000.

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2. Estimation of the Number of Platelets by Phase Microscopy

G. BRECHER and E. P. CRONKITE

The objectives of any counting method for cellular blood elements are two-fold: to obtain a representative sample and to estimate without bias the number of cells in that sample.

Principles of Method: The use of phase microscopy greatly increases the ease with which one can recognize platelets and differentiate them from crystals, amorphous "dirt" or other particles which may contaminate the blood sample.[3,4] The method does not use a stain, which is an advantage since dye precipitates may be mistaken for platelets. The method permits the counting of platelets in either 1:100 or 1:20 dilutions of whole blood. With 1.20 dilutions, adequate numbers of platelets can be enumerated in thrombocytopenic blood with the standard counting chamber to keep the error within acceptable limits.

Apparatus: Flat-bottom counting chamber. "Long working distance" phase condenser with 43X annulus and matching 43X phase objective (if using American Optical equipment, specify "medium dark contrast"). RBC dilution pipettes for 1:100 dilution and WBC pipettes for 1:20 dilution. Dipotassium or disodium ethylenediamine tetraacetate (EDTA, Sequestrene, Versenate). *Optional: siliconed Kahn test tubes; pipette rotors.*

Solution Required: One per cent ammonium oxalate in distilled water. To avoid growth of bacteria, keep stock bottle always in refrigerator, remove a few ml for day's work as needed and discard at the end of day.

Steps in Performance of Method: (1) For best results venous blood should be collected with a 20 gauge needle and siliconed syringe. When difficulty is encountered in entering the vein, 1-2 ml of blood should be aspirated, and the syringe replaced by a second one from which the actual sample is collected (see Chapter 1). The blood can then be transferred to a siliconed Kahn test tube, kept in a beaker with ice water. Dilutions of whole blood are made directly from the test tube with standard RBC or WBC pipettes. Routinely, the following alternatives are used

(a) Blood is collected in a non-siliconed syringe through a 20 gauge needle, and transferred to a test tube or small bottle containing 1 0 to 1 5 mg. of dipotassium or disodium Versenate for each 1 ml. of blood. The test tube or bottle is covered by parafilm and inverted gently 10 times Versenate tubes or bottles for 3-4 cc of blood are conveniently prepared by making a 10 per cent solution of Versenate and permitting 0 04 ml. to dry in the bottom of the tube at temperatures up to 60°C. Versenate dried

above 60°C. dissolves less readily in blood. The dipotassium salt is slightly more readily soluble than the sodium salt. Samples of Versenate blood are quite satisfactory for several hours, provided no difficulty was encountered in entering the vein. Unsatisfactory collection of a sample usually manifests itself in clumping of platelets (see below). For dog blood, the Versenate technic is preferable to direct sampling.[7]

(b) Capillary blood may be diluted directly from a clean finger puncture.

(2) Blood, either from the finger or the test tube, is drawn up to the 1 mark of a red blood cell pipette and one per cent ammonium oxalate to the 101 mark, giving a 1:100 dilution. For thrombocytopenic blood a 1:20 dilution is made in a WBC pipette. Pipettes may be shaken by hand or kept rotating in one of the commercially available pipette rotors until the chambers can be conveniently filled and counted. Rotation of pipettes for as long as 8 hours does not affect the counts.

(3) A hemocytometer is filled in the usual fashion, except that a No. 1 or 1½ cover slip is used rather than the standard hemocytometer cover glass of 0.4 to 0.6 mm. thickness. The use of a thin cover slip is essential in phase microscopy, and its use does not lead to an increase in the error of the count. The counting chamber must have a flat bottom, because a concavity in the bottom of the counting chamber vitiates the phase effect.

(4) The chamber and a wet piece of cotton are covered by a Petri dish for 10 to 15 minutes to allow settling of the platelets and to prevent drying of the preparation.

(5) Ordinarily, platelets are counted in 10 blocks of small squares (as for RBC counts), 5 blocks being counted in each half of the chamber. The total number of platelets so counted times 2500 gives the platelet count per cu mm. A 43× phase objective with a long working distance condenser and a 10× eyepiece is used. In this procedure, the platelets stand out as individual round or oval dark bodies. On focusing up and down, platelets can be seen to have one or more dendritic processes. Crystals, dirt and bacteria are readily distinguished by their refractility and absence of processes. With even slight experience, it is not necessary to visualize the processes of individual platelets, and the count can be completed quickly, once the platelets have settled out, without more than occasional refocusing. The presence of platelet clumps indicates that incipient clotting has occurred in collecting the samples and a fresh sample must be taken. This occurs but rarely and may be traced to difficulties in venipuncture or delay in adding Versenate (or ammonium oxalate diluent when immediate dilution is used).

If fewer than 25 platelets are present in 5 small squares, all 25 small squares are counted on at least one side of the chamber. If the total num-

TABLE 1 *Errors of Platelet Counts from Venous Blood*

Dilution	If No Platelets in First 5 Squares Number	No Squares to be Counted	Result to be Multiplied by	Total No Platelets Counted	Error (Coeff of Variation)	Total Count Obtained	Actual Platelet *Level is* Within (±2 SD)	A Subsequent Count Indicates a Different *Level if the Change* is Greater Than (±2.8 SD)
1:100	More than 25	10	2,500	400	8%	1,000,000	160,000	220,000
				200	9%	500,000	90,000	125,000
				100	11%	250,000	55,000	75,000
				50	15%	125,000	37,000	53,000
1:100	5-25	50	{500	200	9%	100,000	18,000	26,000
1·20	More than 25	10	{500	100	11%	50,000	11,000	16,000
				50	15%	25,000	7,500	11,000
1:20	Less than 25	50	100	200	9%	20,000	4,000	5,600
				100	11%	10,000	2,200	3,000
				50	15%	5,000	1,500	2,200
				10	30%	1,000	600	900

The last two columns represent 2 SD and 2.8 SD respectively. The "true count" is within 2 SD of the sample count in 95 per cent of the cases. Similarly, if a second count differs by 2.8 SD, this indicates an actual change in the platelet level 95 per cent of the time.

If it is desired to increase the accuracy of the count at the 100-150,000 level, 50 rather than 10 squares should be counted when the first 5 squares contain 25-50 platelets.

Accuracy is doubled by counting 4 simultaneous dilutions and chambers *from the same sample*. Counting 4 or 5 times as many platelets in a single chamber is almost as good, because pipette and chamber errors are small compared with the field error which depends on the total number of platelets actually counted.

ber of cells in 50 small squares (25 on each side) falls below 50, (i e., the platelet level is below 25,000 per cu mm.), repeat counts should be made with 1·20 dilutions in WBC pipettes and cells of 10, 25, or 50 small squares counted

Computations

Number of Small Squares Counted	Dilutions	Number of Platelets Multiplied by
10 (5 each side)	1 100	2,500
25 (one side)	1 100	1,000
50 (25 each side)	1.100	500
10 (5 each side)	1:20	500
25 (one side)	1.20	200
50 (25 each side)	1 20	100

Range of Values The mean normal platelet level of healthy adult American and British males is about 250,000/cu mm[8] There is, however, marked variation between individuals. Ninety-five per cent of healthy males have counts between 140,000 and 440,000. In any given individual, the platelet level usually stays constant over many days, months or even years In females, platelet levels are probably similar, but variations with menstration have been reported. Indirect counts have generally given higher values but these are believed to be due to selection of areas counted, e.g , central areas of wet smears[5]

Errors of the Method· The unavoidable errors of the method are the pipette and chamber errors, and the "field error," i e , the statistical error due to counting a limited number of platelets in the chamber[1 5] The expected total error (coefficient of variation) due to combined field, pipette and chamber errors is less than 11 per cent when at least 100 platelets are counted, about 15 per cent when 40 platelets are counted and 30 per cent when 10 platelets are counted These errors have been verified experimentally for venous blood[8] When capillary blood is used, the average count is 2 5 per cent lower, a negligible systematic bias However, the total error is about twice as large than when venous blood is used,[3] probably because of variation of the platelet level in successive drops of capillary blood. Table 1 gives ranges of error and significant differences between two successive counts for venous blood samples.

Precautions: The counting chamber should be scanned for presence of platelet cl[illegible] or undue delay between [illegible] e of platelet clumps pre- [illegible] collected

Difficulties in recognizing platelets in the counting chamber are usually due to failure to clean the chamber and cover slip meticulously, or to faulty adjustment of the phase microscope.

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3. *Preparation of Suspensions of Intact Platelets*

E. P. CRONKITE, G. BRECHER and J. FURTH

Suspensions of platelets prepared by the method of Dillard et al.[1] are "intact" in the sense that 50–90 per cent of such platelets will circulate for some hours, and up to 50 per cent for 24 hours in thrombocytopenic recipients These platelets, however, are not necessarily undamaged. The observations of Lawrence and Valentine[2] indicate that the survival of cross-transfused cat platelets in a thrombocytopenic cat is 4 days. Odell et al.,[3] using tagged platelets, found the survival of platelets in the circulation of normal rats to be 5 days Under similar experimental conditions, the platelets from suspensions prepared by Dillard's method did not circulate longer than 48 hours as a rule Platelets in the circulation are probably flat discs which appear lancet-shaped only when seen on edge, and are presumably free of "pseudopodia" or "spicules."[4] Platelets in sequestrene, when observed under the phase microscope are occasionally seen to be flat discs However, as a rule, varying numbers are elongated and have pseudopodia The number of discs decreases rapidly and after a few hours all have pseudopodia

Apparatus Required Refrigerated centrifuge, set at 3–5°C Siliconed receptacles for whole blood and platelet suspension, sizes depending on volumes to be processed. Fifteen- or 17-gauge electropolished "Fenwal" laminar flow needles. "Nonwettable" plastic tubing. Siliconed pipets

Transfer sets consisting of a suitable length of siliconed glass tubing attached to a plastic tubing and a 5 ml. syringe. The free end of the glass tube is sealed and provided with a lateral opening, about 1 mm. in diameter

as close as possible to the sealed end of the tube. All glassware made pyrogen-free by exposure to 170°C. for 2 hours. Plastic tubing made pyrogen-free by washing with super-oxydol and thorough rinsing in pyrogen-free water

Solution Required 1 to 1.5 per cent Na_2 sequestrene (Ethylene-diamine-tetraacetate, EDTA) in 0.7 per cent sodium chloride in pyrogen-free water, adjusted to pH 6 5 by addition of NaOH.

Procedure: (1) Bleed dogs under pentothal anesthesia from unexposed femoral artery through "Fenwal" needles and plastic tubing into a chilled siliconed bottle containing 10 ml. of sequestrene solution for each 100 ml. of blood In man, phlebotomy with similar needle and tubing, gravity flow are employed. In small laboratory animals, a satisfactory suspension may be obtained after suitable anesthesia by cardiac puncture, with or without exposure of the heart, with needles of suitable size, and collection of blood by very gentle suction into siliconed syringe containing prescribed amount of sequestrene solution. Intravenous injection of heparin before bleeding or exsanguination has proved useful in rats and increases the platelet yield. The amount of sequestrene may be reduced under these circumstances

(2) Centrifuge at 30 g for 50 minutes to sediment RBC (400 to 500 rpm in an International centrifuge with 4 Dural cups for 200 ml bottles.)

(3) Transfer plasma into second siliconed centrifuge bottle or tube. For this purpose, the glass tube of the "transfer set" is kept near the bottom of the plasma layer, 2 to 3 mm. above the RBC layer The syringe is held below the level of the plasma layer, and plasma aspirated until it nearly reaches the tip of the syringe. The plastic tubing is momentarily clamped, the syringe removed, and the plasma allowed, by syphon effect, to flow into the clean siliconed centrifuge tube or bottle.

(4) Centrifuge plasma at 300 g for 45–60 minutes (1500 rpm in an International centrifuge with 4 Dural cups for 200 ml. bottles.)

(5) Decant rapidly all but a small amount of plasma.

(6) Resuspend packed platelets in small amount of plasma by repeated aspiration and expulsion of plasma in siliconed pipette Rather vigorous loosening of packed platelets from the bottom of the centrifuge bottle by "scraping the bottom" with tip of pipette may be required.

Precautions and Sources of Error. The method must be suitably modified to insure sterility if it is to be used in man.* Platelet groups very likely exist in man and possibly in other mammals, and immunization against homologous platelets is likely to occur with suspensions prepared from cross-matched blood. The method has not been subjected to a critical evaluation of the importance of refrigeration to 3–5°C. nor the importance of pyrogen-free technic.

The speeds and times of centrifugation were evaluated and are those found satisfactory in preparation of suspension of dog platelets for transfusion into thrombocytopenic dogs. There are individual variations in the rate at which platelets are sedimented by centrifugation in bloods from different animals or from different bleedings. In some cases recentrifugation may improve the yield Probably, isotonic Na_2 sequestrene (5 per cent in distilled water, 3 ml. per 100 ml. of blood) could be substituted for the solution used by Dillard et al[1] The addition of glucose in concentration similar to that in ACD solution does not interfere with the yield and makes it possible to collect and separate platelets and red blood cells simultaneously. Whether glucose improves platelet survival has not been tested. Only 50–90 per cent of the platelets prepared by Dillard's method are "viable" and are not necessarily equivalent to platelets in the circulation or even platelets in whole blood collected in EDTA.

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* This has recently been accomplished by Gardner, Howell and Hirsch,[5] using a closed system of special plastic bags and transfer sets made by Fenwal Laboratories, Framingham, Mass

4. Separation of Platelets from Blood

L. M. TOCANTINS

The anticoagulants most commonly employed for this purpose are citrates and the oxalates. To keep the platelets intact, the collection of blood should be as rapid as possible and any contact with wettable surface avoided. If it is desired to dilute the blood as little as possible, concentrated solutions of anticoagulant (20 per cent sodium citrate or 25 per cent potassium oxalate) may be used, but unless the blood is collected rapidly and mixed with the anticoagulant at once, many platelets are altered in these solutions

In Man: Place the amount of anticoagulant solution necessary to keep the blood from clotting in the syringe and wet the sides of the syringe thoroughly with it. If the solution is sterile, wet the interior of the needle as well. It is advisable to use an excess of an isotonic solution of the anticoagulant and a syringe of 50 ml capacity With a sharp needle of short shank and wide bore, puncture the vein and draw the blood swiftly into the solution. Collect approximately 30 ml , empty the contents into two plastic or siliconized rounded bottom glass centrifuge tubes in each of which there has been placed approximately 25 ml. of a mixture of citrate (or oxalate) and physiologic solution of sodium chloride. Mix thoroughly by pouring from one plastic or siliconized centrifuge tube into the other Centrifuge at about 1,000 revolutions per minute for 10 minutes. Pipette off the supernatant fluid, which is of a light pink color, into plastic or siliconized glass, conical bottom centrifuge tubes and centrifuge for an hour at about 2,000 revolutions per minute. Pipette off the supernatant fluid and discard it. The accumulated sediment appears distributed in two layers. a thin layer of red blood cells and, superimposed on it, a thick white layer of platelets, among which are mixed a few white and red blood cells. Cover the sediment gently with approximately 10 ml of cold physiologic solution of sodium chloride By applying suction through a siliconized glass pipet (capillary tubing drawn out to a tip measuring about 1 mm in internal diameter), remove the white layer carefully, beginning with the central portion and gradually working toward the periphery and more deeply toward the packed layer of red blood cells Place the mixture of salt solution and platelets into a 15 ml conical bottom, siliconized glass tube and emulsify gently by aspirating and expelling it with a siliconized dropping rubber bulb pipette. Avoid bubbling. After the solution is free from coarse floating particles and has an even pale blue translucent appearance, centrifuge it again for one hour at 2,000 revolutions per minute or until the supernatant

...marked, remove the supernatant ...

and red blood cells. ... emulsion should be made to make sure that the white and red ... being removed. In pipetting off the white layer, no attempt should be made to remove it entirely, down to the level of the red blood cells; if a white film is left over the packed red cells, most of the white cells will have been eliminated. By this process approximately two thirds of the collected platelets are isolated.

The amount of blood collected may be adjusted, depending on the purpose for which the platelets will be used. Greater amounts may be isolated by having several syringes loaded with the anticoagulant solution and switching from one to another, the needle being left in place until the desired amount of blood is collected

All glassware must be scrupulously clean, the solutions filtered and cold All contact with cotton or thready material must be avoided; such mater... for the bottles containing the solution. ... to allow sufficient time for the various ... platelet emulsion, while not in use, should be kept in the ice chest at a ... erature of approximately 5°C

... isolated from the blood of animals in a ... jugular vein in the unanesthetized ... n a dog weighing approximately 15 kilograms 200 cc of blood ... collected from the heart without any disturbing symptoms The puncture is made with the animal's head immobilized and the body firmly stretched and bound on a board. The syringes are loaded with the solution and laid by the side of the operator Since the puncture is made almost vertically, a brake is placed on the piston to keep the solution from flowing out The needle is left in the heart while syringes are being shifted.

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5. Estimation of the Adhesiveness of Blood Platelets (Method of Wright)

Adapted by R. R. HOLBURN*

Object of the Method: The measurement of the degree to which platelets adhere to certain surfaces.

Principle: Blood platelets in vitro progressively decrease in number, as time following withdrawal increases. Platelets adhere readily to glass surfaces and the rate of disappearance from blood samples in contact with glass serves as an index of their adhesiveness.

Apparatus and Reagents: Beakers and syringes (with non-wettable surfaces, e g , silicone or plastic, or thinly coated with liquid paraffin); special glass tubes with a central bulb for rotating blood samples (see original article); Rees-Ecker diluting fluid containing 2 per cent formalin instead of 0 2 per cent; Bürcher Counting Chamber; red cell pipettes.

Procedure: 5 ml. blood is drawn into a syringe and immediately transferred into a beaker containing 0.2 mg heparin per ml. blood The beaker is gently agitated to mix the contents Within 5 minutes, 2 ml. of the heparinized blood is transferred to a *special glass tube* which is mechanically rotated for 80 minutes. Platelet counts are made on the blood initially and on samples drawn from the rotating tube every twenty minutes The platelet counts are done by the direct wet method, the whole field of the Burcher chamber being counted. In order to prevent adhesion of the platelets to the dry stem of the pipette, the diluting solution is first drawn up to the 0 5 mark and the blood then drawn into this up to the 1 0 mark A control sample from a known normal individual is treated the same way

Calculation. The number of platelets expressed as percentage of the initial count is plotted against the time of rotation in 20 minute intervals. The estimation of platelet stickiness of the unknown blood is estimated by comparison with the control sample under identical conditions

Values Obtained: Normal average 36 per cent of normal platelets remain free (range 31–42 per cent), after 80 minutes of rotation of the tube.

Precautions and Sources of Error (1) Since platelets adhere readily to glass surfaces, the use of non-wettable surfaces is required in the initial steps of the procedure In collecting the platelet counts, for the same reason, it is important that the diluting fluid be drawn into the pipette first, then the blood drawn into it

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* From Lancet *1* 306, 1946

(2) Since the percentage of platelets removed is dependent upon the concentration of anticoagulant present, the procedure should be followed as described. The higher the concentration of anticoagulant, the less adhesive are the platelets.

Discussion: *The progressive fall in platelet counts of blood in vitro is* mainly, if not entirely, due to their adhesion to the glass walls of the container rather than disintegration. The platelets in the cases of hemophilia studied are significantly less adhesive than the normal platelets.

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6. *Induction of Thrombocytopenia in Animals by Ionizing Radiation*

E. P. CRONKITE

Fabricius-Moller[1] clearly demonstrated in 1922 that a severe thrombocytopenia could be induced by a single dose of ionizing radiation in guinea pigs He further demonstrated that shielding of the legs lessened the severity of the thrombocytopenia. The latter study is the key to the successful and *uniform induction of any desired degree of thrombocytopenia in animals.* The exposure to radiation must be uniform over the whole body with a relatively constant depth dose so that all hemopoietic tissues absorbs relatively the same amount of energy The basic considerations necessitate more extensive and frequent calibration of equipment than is usually performed for therapeutic x-ray The field of radiation must be measured carefully in all quadrants to determine the variation in intensity Variation of *greater than ±2 per cent should not be permitted* *With some x-ray* tubes it is necessary to alter the cones or build parabolic filters in order to get large fields of uniform intensity The next physical consideration of prime importance is a constant depth dose in the animal. This is determined by measuring the dose at various depths in material of electron density similar to tissue and of approximately the same size as the animals to be exposed If the fall off in intensity due to absorption and inverse square is greater than 10 per cent it must be compensated for by some or all of the following maneuvers. (a) increase distance from anode to skin (minimize inverse square), (b) add filters to increase effective energy; (c) split the dose half

to each side of the animal. (a) and (b) decrease the dose rate For practical purposes, dose rates less than 6 r per minute are undesirable because the effect at lower rates becomes dependent upon dose rate. In the event that the dose rate becomes too low, the only alternative is to use higher energy radiation

For mice, rats, guinea pigs, and to a limited extent, rabbits, the standard 200, 250, and 400 kvp therapy x-ray machines are satisfactory when some or all of the modifications above are utilized. For larger animals, it is difficult to get sufficiently large fields with satisfactory depth dose and adequate dose rates.

In practice the dose is split equally to both sides of all mammals except the mouse. For the induction of thromocytopenia in dogs of all sizes, the following procedure has yielded satisfactory, reproducible, and quantitative results. (a) 2 0 mev G E. industrial x-ray machine*; (b) radial beam; (c) TSD, 2.0 meters; (d) Dose rate 14 r/min ; (e) HVL 4 3 mm. Pb; (f) Effective energy approx. 500 kev; (g) Turn dogs around after delivering half of dose

The degree of thrombocytopenia and the time of recovery are a function of dose as are mortality and survival time.[2] At higher doses the survival time becomes so short that thrombocytopenia does not develop. For reference purposes the relationship of dose to mortality, survival time, and bleeding tendency is shown in table 1.

With doses that produce survival time of less than 6 days, thrombocytopenia does not develop. Between 150 and 1000 r a definite thrombocytopenia develops. From 500–1000 r the response is similar and maximal. For all doses, the platelet count may trend upwards for a day or so By the 5th day the count is decreasing and minimum levels are attained by 9–12 days and a relatively constant level is maintained for 2–3 weeks, at which time recovery begins or the animals may die at any time from the sequelae of pancytopenia. The characteristic response of the dog is tabulated·

Dose in r	Time to Minimum (Days)	Duration of Steady State Thrombopenia	Average Level of Steady State	Recovery Begins
150	10–12	10–20 days	100,000/mm³	3rd–4th wk
200	10–12	10–20 "	50 000/mm³	" " "
300	10–12	10–20 "	30 000/mm³	" " "
400	9–11	10–30 "	15 000/mm³	4th–5th "
600	9–10	From 10th day to death	0–5,000/mm³	None

* 1 0 mev G E. x-ray machine has worked equally well as has cobalt gamma rays Less powerful machines can be used but reproducibility is less accurate

Table 1: *Untreated Dog Mortality—2m e.v. (Bilateral Technic)*

Dose (r)	No. Exposed	No Survived	No Died	% Mortality	Survival Time in Days Min.	Survival Time in Days Mean	Survival Time in Days Max.	Degree of Bleeding
150	18	18	0	0	—	—	—	±
200	11	8	3	27.3	12	16	21	+
300	15	10	5	33.3	15	19.6	23	2+
350	8	4	4	50.0	10	14.9	20	3+
375	3	0	3	100.0	14	19.0	24	3+
400	26	4	22	84.6	6	17.6	40	4+
450	4	0	4	100.0	14	14.5	15	4+
500	11	0	11	100.0	7	10.0	12	4+
600	49	0	49	100.0	4	11.9	21	4+
800	4	0	4	100.0	9	11.8	17	4+
1000	7	0	7	100.0	3	7.0	9	2+
1500	6	0	6	100.0	0.5	3.1	4	0
2000	6	0	6	100.0	3	3.8	4	0
3000	6	0	6	100.0	3	3.0	3	0

Thrombocytopenia can also be induced by chronic exposure to radia- t [illegible] level to be a very sensitive index [illegible] of 600 r without as severe a [illegible]

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7. Estimation of Platelet Agglutinating Activity by a Semiquantitative, Macroscopic Technic

K. M. BRINKHOUS, R. H. WAGNER and R. G. MASON

Object: To detect and quantitate platelet agglutinating agents by determining both the time required for macroscopic agglutination of washed platelets and the degree of agglutination.[1]

Principle: Such diverse agents as recalcified plasma, serum, thrombin, adenosine diphosphate, and polylysine will induce macroscopically visible agglutination of washed platelets The time required for agglutination varies inversely and the degree of agglutination varies directly with the concentration of the agglutinating agent in this test. Important variables which should be controlled in the test are pH, ionic strength, temperature, cation concentration, platelet concentration, and concentration of agglutinating agent.

Materials: (1) Clean, scratch-free, 10 x 75 mm. glass test tubes in which the agglutination test is performed. (2) Several stopwatches graduated in tenths of a second. (3) A strong light source, such as a 100 watt bulb with a reflector, for observation of platelet agglutination. (4) A phase contrast microscope and counting chamber for performance of platelet count. (5) Silicone-coated glassware treated with SC-87 Dri-Film (General Electric). The quantity and size of glassware items are dependent upon the volume of material to be processed. (6) A centrifuge equipped with both angle and horizontal heads

Reagents: 1. Isotonic saline This is 0.154 M NaCl.

2. Ethylenediamine tetraacetic acid (EDTA). An isotonic stock solution, pH 7.38, is prepared by dissolving 15.2 Gm. of the disodium salt (Geigy) and 17.8 Gm. of the trisodium salt in water to give 1 liter This stock solution is diluted with isotonic saline before use, as indicated below.

3. Imidazole buffer. A 0 084 M solution is prepared by dissolving 1.72 Gm. imidazole in 90.0 ml 0.1 N HCl, adjusting pH to 7 4, and diluting to 300 ml. with isotonic saline.

4 Citrated saline. Mix one volume 0 11 M sodium citrate with 19 volumes 0 154 M NaCl.

5. Washed platelet suspension. Isolation and washing of platelets may be carried out at room temperature (28°C.) or at 4°C All glassware, including pipettes and syringes, should be silicone-coated Whole blood, obtained by the two syringe technic, is drawn into an equal volume of EDTA solution made by adding 9 parts saline to one part stock EDTA solution After centrifugation for 8–10 minutes at 100 g using the horizontal head, the supernatant platelet-rich plasma is carefully aspirated to

prevent contamination with sedimented erythrocytes or leukocytes. Collection of platelet-rich plasma is best performed with a long aspirating needle (16 gauge) and a silicone-coated syringe. The platelet-rich plasma is centrifuged for 5 minutes at 2075 g using the angle head. The platelet-poor supernatant is discarded, and the platelet pellet is resuspended in 1/4 to 1/5 volume EDTA solution made by adding 99 parts saline to one part stock EDTA solution. Resuspension is performed by gently drawing the fluid repeatedly into a 2 ml. pipette. Release of fluid from the pipette is accomplished by gravity flow, with avoidance of bubble formation. The platelet suspension is centrifuged for 5 minutes at 2075 g, the supernatant discarded, and the platelet pellet resuspended in 1/4 to 1/5 volume citrated saline. This step is repeated, and the platelets suspended in citrated saline are centrifuged 7 minutes at 90 g to remove any remaining erythrocytes. The platelet suspension is carefully decanted and the erythrocyte button discarded. One part of this platelet suspension is diluted with 39 parts citrated saline, and the diluted suspension used for determination of the platelet count. The remainder of the suspension is diluted with citrated saline, as indicated by the platelet count, to a concentration of 1×10^6 platelets/mm.[3]

6. Cation solutions. Stock solutions of 0.108 M $CaCl_2$, $MgCl_2$, and $MnCl_2$ are approximately isosmotic with 0 154 M NaCl Appropriate dilutions of these stock solutions are made with isotonic saline.

Steps in the Test. The material to be tested for platelet agglutinating activity is added to a suspension of washed platelets. The following is a sample test system

1 part material to be tested
1 part 0 0108 M $CaCl_2$ or 0.0027 M $MgCl_2$
1 part 0 084 M imidazole buffer
1 part 0 154 M NaCl

These reagents are mixed well at 28°C and the standard test is performed by mixing the following

0 4 ml above test mixture
0.1 ml. platelet suspension

A stopwatch is started immediately The test is observed against a brightly illuminated background The contents of the tube must be agitated rhythmically by gentle thumping of the tube, since platelet aggregates are best observed in the film of fluid falling down the wall of the tube farthest from the observer. The moment of appearance of macroscopically visible platelet aggregates is recorded as the agglutination time Two minutes after addition of test mixture to platelets, the aggregates are graded macroscopically on a scale of +, ++, +++, and ++++. Microscopic examination of platelet aggregates will reveal about 5 platelets per + aggregate, 10–25 per ++, 30–100 per +++, and over 100 per ++++ aggregate Tests which

appear negative macroscopically should be examined microscopically before final conclusions are drawn.

Control tests should be performed in which the platelet suspension is tested separately with each of the following: saline, imidazole buffer and saline, and cation solution and saline. Suspensions of platelets which agglutinate in any of these control tests are unsuitable for use. Mixtures consisting of only the material to be tested for agglutinating activity and saline may induce platelet agglutination if no cationic cofactor is required. Similar mixtures may induce platelet agglutination even though a cationic cofactor is required, if the material to be tested for agglutinating activity contains cations in sufficient concentration, e.g., serum.

Expression of Results: Results are expressed simply as agglutination time and degree of agglutination, e.g., 7.0 second, 4+.

Alternate Procedures. The test system is flexible and may be tailored to the needs of the individual investigator. For example, if plasma is to be tested for potential thrombin agglutinating activity, it is first activated with thromboplastin and calcium.[2] In the second stage of this two-stage test system, the activated plasma is added to the platelet suspension.

Precautions. (1) Several platelet agglutinating agents, such as thrombin,[3] thrombocyte agglutinating factor (TAg), and adenosine diphosphate, appear to require certain cationic cofactors for induction of agglutination. It is wise to test any agglutinating agent in the presence of several different concentrations of the divalent cations of calcium, magnesium, or manganese.

(2) Platelet agglutination is difficult to observe macroscopically at final platelet concentrations of less than $1 \times 10^5/mm^3$,[3] and test systems should be designed accordingly. In the standard test system given above, the final platelet concentration is $2 \times 10^5/mm^3$.

(3) The pH of test systems should be kept at near physiologic values.

(4) It is essential that the procedures for isolating and washing platelets be carried out as rapidly as possible. Delays during any of the preparative steps generally result in decreased final platelet yield.

(5) The final platelet suspension is stable for over 3 to 4 hours, if kept at melting ice temperature. If chilling of the platelets is not desired, the final suspension is often stable for 2 to 3 hours at room temperature.

(6) Agglutination of platelets during the preparative steps or in control tests with cation frequently can be attributed to incipient thrombin formation. Careful venepuncture technic is essential for successful platelet preparation.

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8. Methods for the Estimation of the Life Span of Platelets

T. T. ODELL, JR. and T. P. McDONALD

A variety of methods have been used to determine the life span or survival time of blood platelets of various species[1] The methods most commonly used in human blood platelet work have been the measurement of the disappearance of labeled platelets from the peripheral circulation after (1) injection of P^{32}-labeled diisopropylfluorophosphate (DFP^{32}), which combines immediately and irreversibly with esterase enzymes on circulating platelets and other blood cells;[2] and (2) transfusion of platelets labeled in vitro with $Na_2Cr^{51}O_4$.[3] A third method used by us, primarily in laboratory animals, measures the length of time taken to reach constant platelet radioactivity during daily injection of $Na_2S^{35}O_4$.

Injection of P^{32}-Labeled Diisopropylfluorophosphate (DFP^{32})

1 *Object of Procedure* To determine the life span of blood platelets.

2. *Principle of Procedure* DFP^{32} combines immediately and irreversibly with esterase enzymes on circulating platelets and other blood cells. If injected intravenously, the pool of DFP^{32} is available for a relatively short time after injection because of rapid combination and excretion, and peak platelet activity is usually reached within 2 hours after injection Since only diisopropylphosphate, a substance that is rapidly excreted and does not combine with blood cells, has been found after cell destruction, there is no apparent problem of reutilization Furthermore, because the disappearance curve from peripheral circulation of DFP^{32}-labeled erythrocytes and platelets has usually been described as linear, elution of the label from cells apparently does not take place Platelets must be essentially free of erythrocytes and leukocytes before determining their radioactivity, for these also are labeled with DFP^{32}

3. *Reagents and Apparatus Required*· (a) DFP^{32} is purchased as a [illegible] (about 150-200 [illegible]) Dose per individual [illegible] 100 [illegible] Dose in rats is 0.2 to 0 5 mg. of DFP^{32} per Kg of body weight, specific activity of 100 to 200 μc./mg.

(b) Equipment for collecting blood and segregating platelets therefrom, including siliconized glassware, and a centrifuge.

(c) Counter and scaler for measuring P^{32} radioactivity of samples (e.g , a thin-window, gas-flow, proportional counter) Planchets on which to place radioactivity samples.

4. *Steps in Procedure.* (a) Give a single injection intravenously (over 3- to 5-minute period in man) DFP^{32} also may be given intramuscularly to man and animals

(b) Collect blood samples 2 to 4 hours after injection and daily until platelet radioactivity has declined to essentially zero (some alternate days can be omitted). Under conditions of good platelet yield and a fairly sensitive counter of radioactivity, 15 ml blood samples will be sufficient. Since the first samples will have the highest activity, it can then be determined whether the volume of later samples needs to be increased. In smaller laboratory animals, blood is obtained by exsanguination in order to obtain a sufficient quantity Collect into siliconized glassware containing EDTA (a 1 per cent solution of disodium ethylenediaminetetraacetate dihydrate in 0 7 per cent saline) as the anticoagulant in a quantity of approximately 10 per cent of the blood volume to be collected.

(c) Separate platelets from blood by differential centrifugation (methods described elsewhere in this book) Wash platelets twice by suspending platelet button in saline and centrifugation

(d) Resuspend platelets in a small volume of saline. Make numerical counts of platelets, white blood cells, and erythrocytes (no more than one red or white cell per 1000 platelets) Place a known volume (about 0 2 to 0 5 ml) of platelet suspension on planchet containing a circle of lens paper Add 0 1 ml of 30 per cent NaOH to samples on planchet and heat in oven at 100°C for 30 minutes to hydrolyze any free DFP to non-volatile diisopropylphosphate Dry and count radioactivity

5 *Method of Expressing Results*· Express results as counts per minute per average platelet

$$\frac{\text{counts/min of sample minus background}}{\text{sample vol} \quad (\mu L)} = \text{activity}/\mu L. \qquad (1)$$

$$\frac{\text{activity}/\mu L.}{\text{number of platelets/mm}^3\ (\mu L)} = \text{activity/average platelet} \qquad (2)$$

Plot values on graph with platelet radioactivity on ordinate and time in days on abscissa.

6 *Life Span Determination* The point at which a straight line drawn through the points on the graph intercepts the abscissa gives the value of the life span in days.

7. *Normal Values of Platelet Life Span with this Method*

Man	8-14 days
Calf	10 days

In Vitro Labeling of Platelets with Cr^{51}

1. *Object.* To determine the life span of blood platelets

2. *Principle of Procedure* Platelets can be labeled by incubating them for a brief period in vitro with $Na_2Cr^{51}O_4$ Peak platelet activity in the peripheral circulation is reached within 24 hours after transfusion, the delay apparently being due to temporary sequestration of platelets. The platelet loss curve after the first day is now described as a two-component exponential curve that may signify random loss of platelets from the circulation. Elution may contribute to the exponential character of the curve, because elution of Cr^{51} from platelets has been demonstrated under in vitro conditions In addition, release of sequestered platelets may affect the shape of the decay curve. In spite of some uncertainty about interpretation of the shapes of the curves, those curves that depict abnormal survival times can be distinguished from normal ones, and the method thus provides a useful diagnostic tool The Cr^{51} technic may be advantageous in cases where the patient has too few platelets of his own to make autologous labeling and repeated sampling practical, thus making it necessary to label and transfuse platelets from a homologous donor

3 *Reagents and Apparatus Required* (a) Sterile $Na_2Cr^{51}O_4$

(b) Plastic bag equipment for collecting, labeling, and transfusing donor platelets Refrigerated centrifuge EDTA, an anticoagulant Equipment for collecting samples of blood of recipient and separation of platelets from it (siliconized syringes, pipettes, centrifuge tubes, centrifuge; anticoagulant)

(c) Counter and scaler for counting gamma emissions (well-type scintillation counter)

(d) Triton (Entsufon, WR1339) may be used to aid resuspension of platelets from a button, a 5 per cent solution of ascorbic acid

(e) Equipment and materials for making numerical counts of platelets (phase microscope, etc)

4 *Steps in Performance of Procedure* (a) Collection of donor blood and in vitro labeling of platelets *Collect 500 ml of donor blood into a plastic bag containing EDTA Centrifuge (325 g, 15 minutes) in cold to*

obtain platelet-rich plasma, and transfer later to another plastic bag (Triton may be used to facilitate resuspension of platelets) by means of a piercing coupler. Obtain platelet button by centrifugation (1000 g; 30 minutes), withdraw plasma and save 10 ml. Add 10 ml of 0.2 per cent Triton in saline and resuspend platelet button Introduce 300 μc. of $Na_2Cr^{51}O_4$, specific activity of 20 μc/μg. Mix and incubate at room temperature for 15 minutes Centrifuge (1000 g; 30 minutes), remove suspending medium (radioactive platelet-poor plasma), and resuspend platelets in 15 ml of non-radioactive platelet-poor plasma obtained earlier and 15 ml. of 0 2 per cent Triton in saline. Add 100 mg. of a 5 per cent ascorbic acid solution to discourage binding of excess $Na_2Cr^{51}O_4$ by red cells of recipient Infuse platelet suspension into recipient.

(b) Collection of platelets of recipient and measurement of radioactivity. Twelve to 20 ml. blood samples are collected from the recipient daily until the radioactivity disappears. Platelets are separated from the samples by differential centrifugation or sedimentation using siliconized glassware (see elsewhere in book) and washed in 10 ml. of saline The platelet button may then be resuspended, a numerical platelet count made, and a known volume of the suspension counted in a well-type scintillation counter. An alternate procedure is to measure the radioactivity of the final platelet button directly. This requires that the efficiency of platelet collection remain essentially constant.

5 *Methods of Expressing Results* (a) Described under the DFP^{32} method, or

(b) Counts per minute per platelet button

In each case, plot platelet radioactivity on ordinate and time in days on abscissa

6 *Comparison* Compare curves of normal subjects with those of subjects that have a platelet dyscrasia

7 *Normal Values of Platelet Survival* A life span in man of 8 to 10 days has been reported with this method. If, however, the curve actually describes a random loss of platelets, platelet survival should be described in terms of half-life (2 to 3 days) or maximum survival (about 8 to 10 days). In any case, abnormal curves can be distinguished from normal ones.

DAILY INJECTION OF $NA_2S^{35}O_4$

1. *Object of Procedure:* To determine the life span of blood platelets.

2. *Principle of Procedure:* Sulfate is incorporated into a sulfated mucopolysaccharide of blood platelets, apparently in the megakaryocyte stage before release of platelets into the circulatory system Daily injection of $Na_2S^{35}O_4$ maintains a relatively constant pool of label Frequent measurement (preferably daily) of the radioactivity of the circulating platelet popu-

lation reveals a steadily increasing radioactivity level for several days, fol- ... indicates, according to our interpretation, ... labeled platelets entering the circulation are ... ng the circulatory system. This technic is not useful in ruminants whose intestinal flora convert the sulfate sulfur to sulfhydryl sulfur.

3. *Reagents and Apparatus Required:* (a) Sterile, carrier-free $Na_2S^{35}O_4$ (about 750 μc/ml.) is commercially available. Doses used in animals have been 0.1 to 1.0 μc./gm. body weight per day. Much smaller doses have been used in human patients, about 8 to 25 μc/Kg. per day.

(b) Equipment for use in collecting blood and separation of platelets therefrom (siliconized syringes, pipettes, centrifuge tubes; centrifuge; anticoagulant)

(c) Counter and scaler for measuring radioactivity of platelets and plasma One can use a windowless, gas-flow proportional counter or a liquid beta scintillation counter (authors use the former). Planchets or bottles can be used for radioactivity samples.

(d) Polyvinylpyrrolidone (PVP), a plasma expander, or saline (5 parts of saline to 8 parts of blood) may be added to the blood before centrifugation to improve yield and purity of platelets

(e) Equipment and materials for making numerical counts of platelets (phase microscope, etc)

4 *Steps in Performance of Procedure:* (a) Inject isotope intraperitoneally at same time (morning) each day to maintain a pool of label.

(b) Collect blood samples daily, 24 hours after previous injection of isotope, into siliconized syringes containing an amount of the anticoagulant EDTA (a 1 per cent solution of disodium ethylenediaminetetraacetate dihydrate in 0 7 per cent saline may be used) equal to approximately 10 per cent by volume of the amount of blood to be collected Keep blood chilled (about 5°C)

Laboratory animals: Kill animals serially by exsanguination (ether or Nembutal anesthesia) to collect sufficient platelets for radioactivity assay Animals may be injected with a heparin-Nembutal-saline solution before exsanguination to help prevent clotting and to provide anesthesia

Humans Collect a 15–30 ml blood sample (size of sample depends on isotope dose, platelet yield, and efficiency of counting method) immediately before next injection of isotope The injections and sampling should be continued for 3 to 6 days after reaching the plateau level (make estimate before beginning experiment)

(c) Separate platelets from blood (methods described on page 59 in this book) and wash the platelets twice by suspending the platelet button in physiologic saline and centrifugation

(d) Resuspend platelets in a small volume of saline. Make a numerical platelet and white blood cell count, and if a gas-flow proportional counter is used, place a known amount of platelet suspension, plasma, and washes on planchets and dry Duplicate samples are desirable. Ascertain by clarity of the sample or by observation under a microscope that essentially all platelets have been removed from plasma and washes before sampling (recentrifugation may be necessary).

5. *Methods of Expressing Results* Express results as counts/minutes per average platelet, using equations (1) and (2). Plot values on graph paper with platelet radioactivity on ordinate and time in days on abscissa. The platelet radioactivity points of the first several days will determine an ascending straight line. The level of the plateau may be determined by averaging all values of platelet radioactivity after regular increase in radioactivity ceases

6 *Life Span* The increase of platelet radioactivity per day divided into the value of plateau radioactivity gives platelet life span

7 *Normal Values of Platelet Life Span with this Method*

Man	9-10 days
Rat	4.5 days
Mouse	4 days

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lation reveals a *steadily* increasing radioactivity level for several days, fol- [illegible] indicates, according to our interpretation, [illegible] labeled platelets entering the circulation [illegible] ing the circulatory system. This technic is not useful in ruminant [illegible] whose intestinal flora convert the sulfate sulfur to sulfhydryl sulfur.

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(b) Equipment for use in collecting blood and separation of platelets therefrom (siliconized syringes, pipettes, centrifuge tubes; centrifuge; anticoagulant).

(c) Counter and scaler for measuring radioactivity of platelets and plasma. One can use a windowless, gas-flow proportional counter or a liquid beta scintillation counter (authors use the former). Planchets or bottles can be used for radioactivity samples.

(d) Polyvinylpyrrolidone (PVP), a plasma expander, or saline (5 parts of saline to 8 parts of blood) may be added to the blood before centrifugation to improve yield and purity of platelets.

(e) Equipment and materials for making numerical counts of platelets (phase microscope, *etc*)

4 *Steps in Performance of Procedure* (a) Inject isotope intraperitoneally at same time (morning) each day to maintain a pool of label

(b) Collect blood samples daily, 24 hours after previous injection of isotope, into siliconized syringes containing an amount of the anticoagulant EDTA (a 1 per cent solution of disodium ethylenediaminetetraacetate dihydrate in 0 7 per cent saline may be used) equal to approximately 10 per cent by volume of the amount of blood to be collected Keep blood chilled (about 5°C).

Laboratory animals Kill animals serially by exsanguination (ether or Nembutal anesthesia) to collect sufficient platelets for radioactivity assay Animals may be injected with a heparin-Nembutal-saline solution before exsanguination to help prevent clotting and to provide anesthesia.

Humans Collect a 15–30 ml blood sample (size of sample depends on isotope dose, platelet yield, and efficiency of counting method) immediately before next injection of isotope The injections and sampling should be continued for 3 to 6 days after reaching the plateau level (make estimate before beginning experiment)

(c) Separate platelets from blood (methods described on page 59 in this book) and wash the platelets twice by suspending the platelet button in physiologic saline and centrifugation.

1.	Round or circulating forms	3.5%
2.	Early process formation	6.2%
3	Full process formation	76.6%
4.	Aggregation (early viscous metamorphosis)	6 6/100 platelets counted
5.	Intermediate forms	4.1%
6.	Expanded or spread forms	9.6%

Precautions and Sources of Error: Correlative studies of the plasma coagulation factors are conducted to establish the normality of the platelet environment, or to document deficiencies or abnormalities in the platelet environment. Ultrastructural platelet disease can then often be established by the demonstration of marked deviations from the normal differential platelet counts, for example figure 1 depicts the failure of the platelets in immune type thrombocytopenic purpura to achieve full process formation and figure 2 illustrates a persistent predominance of abnormal viscous metamorphosis or platelet aggregation in patients with pernicious anemia in remission Standardization of the time allowed for platelet transformation before fixation is mandatory because of the time-dependency of this transformation.

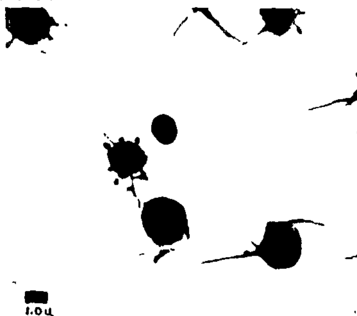


FIG 1 —Typically depressed process formation of platelets in immune type thrombocytopenic purpura Heparinized, OsO_4 fixation.

9. A Method for the Electron Microscopy of Platelets

J. W. REBUCK and J. M. RIDDLE

The rapid and progressive structural transformation of platelets in shed blood often requires a method of study permitting observation of the surface activity and granular release of the entire platelet. A modification of the method originally suggested by Braunsteiner and his associates[1] permits such study and is utilized as follows.

Procedure: Fifteen ml. of peripheral blood are obtained by venepuncture through a sterile, siliconized 19 gauge needle and a siliconized 20 ml. syringe As soon as possible the blood is introduced into a siliconized rectangular vessel which contains 1 ml of liquid heparin (1000 USP units per ml.). Then a slide, previously coated with a 1 per cent of Formvar, is introduced so that the blood covers the slide. The vessel containing the slide is placed horizontally into a 37°C constant temperature oven and a stop watch is started This system is incubated at 37°C for exactly 8 minutes. The slide is withdrawn, washed briefly with Tyrode's solution, heated at 37°C, and fixed in a solution of 1 per cent buffered osmium tetroxide for 15 minutes at room temperature The slide is next washed with distilled water at room temperature and allowed to air-dry.

Final specimen mounting can be facilitated as follows The slide prepared as above is scraped at both ends and at each side with a sharp scalpel to remove the thickened portions of the Formvar film, thus freeing the film from the glass slide Representative areas on the film are selected by use of the phase microscope and the specimen screens are mounted over the desired regions After exhaling lightly onto the film's surface, the entire Formvar film, including the specimen screens, is covered with Scotch Tape. This tape is pressed firmly against the slide and each specimen screen When the Scotch Tape is removed the now adherent Formvar film with the trapped specimen screens is also removed A careful dissection of the Formvar film about the screen's circumference and removal of the screen from the tape allows the specimen, interposed between the screen and Formvar film, to be studied The yield of suitable material with this method of mounting should run well over 95 per cent

Results Differential platelet counts under the electron microscope from nine normal controls[2] reveals six stages of structural transformations as follows.

10. Estimation of Platelet Factor 3 Activity

S. A. JOHNSON

Object: To measure the ability of isolated platelets, when mixed with plasma components, to convert prothrombin preparations to thrombin. This activity of platelets most likely takes part in the prothrombin consumption time, and is measured by the platelet thromboplastin generation. The later test, however, is not specific for the lipoprotein called platelet factor 3 but functions equally well with isolated lipids from platelets, brain and soybeans as well as other tissues. The procedure of Alkjaersig, Abe and Seegers seems to be equally satisfactory for platelet factor 3 of whole intact platelets, platelet extracts or purified platelet factor 3.

Principle: The estimation of platelet factor 3 depends on the conversion of a large predetermined quantity of purified prothrombin to thrombin by the interaction of platelet factor 3 from a known number of platelets and an excess of plasma factor(s) such as the antihemophilic factor or Christmas factor or the substitution of an antihistamine such as Benadryl The resulting thrombin is diluted and measured quantitatively by the clotting of fibrinogen according to Seegers and Johnson (p. 229).

Preparation of Reagents and Laboratory Ware. (1) *Purified prothrombin:* This reagent can be obtained from Dr. Heron O. Singher, Ortho Research Foundation, and is suspended in 10 per cent imidazole buffer in saline to give a prothrombin concentration of about 3000 units per ml. Prothrombin is stable in the frozen state in this buffer, but repeated thawing and refreezing should be avoided. The prothrombin is prepared according to the method of Seegers and possess a specific activity of about 20,000 units per mg tyrosine.

(2) *Benadryl* (diphenhydramine hydrochloride): This replaces platelet cofactors such as the antihemophilic factor. A synthetic compound is advantageous because solutions of this antihistamine are very stable For this procedure a 0 1 Gm per cent solution in physiologic saline is used. Benadryl hydrochloride can be purchased from Parke, Davis & Co , Detroit, Mich

(3) *Platelets* It is difficult to isolate platelets with any significant purity from less than 100 ml of blood. The experimental blood pack of Fenwal Laboratories, Framingham, Mass , Y-1682, is used. These plastic bags contain 15 ml of ACD solution formula "A" Other anticoagulants such as sodium oxalate or disodium ethylenediaminetetraacetate dihydrate solution (EDTA) may be used The isolated platelets are counted by the Brecher et al method and frozen at a concentration of 500,000 cu mm

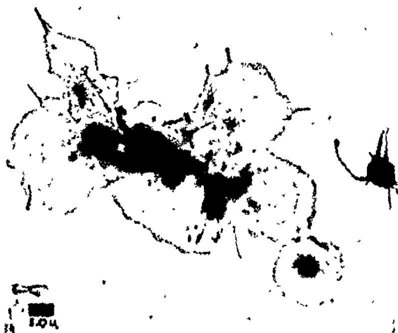


FIG 2.—Pernicious anemia in remission Persistence of abnormally increased spread aggregates Note retention of granulomeres Heparinized, OsO_4 fixation

Minor modifications of this method for the electron microscopic study of platelet-antibody relationships, stored platelets and platelet-fibrin relationships without the introduction of an anticoagulant have been described in a prior work [3] Membrane and granular ultrastructural changes may be studied in ultrathin sections by the method of Schulz, Jürgens and Hiepler,[4] to which the reader is referred

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Platelet suspension (300,000 cu mm)	0 25 ml
Benadryl	0 25 ml.
Calcium in imidazole	0.25 ml
Thrombin 100 units/ml	0 25 ml
Prothrombin 3000 μ/ml.	0 50 ml
Total volume	1.50 ml

Start the stop watch at the same time the prothrombin is blown in and place the reaction tube in a water bath at 28°C. At intervals of perhaps 10 minutes, 25 minutes, 40 minutes and 60 minutes, aliquots of 0 1 ml. should be removed from the incubation tube and, after suitable dilution in saline, be added to fibrinogen in the same manner as in the thrombin assay of Seegers and Smith. The concentration of thrombin is determined accurately.

Calculations: The prothrombin is diluted 3 times in the reaction tube so that a final concentration of 1000 units per ml is obtained and therefore 1000 units per ml of thrombin represents complete activation. The resulting thrombin is diluted 5 times in the clotting tube and if a 200 fold dilution (0 1 ml in 20 ml of saline) results in a 15-second clot, 200 × 5 × 1.00 = 1000 units/ml. of thrombin means that all the prothrombin placed in the reaction tube was converted to thrombin by 0.25 ml. of platelet suspension, concentration 300,000 cu mm. This platelet suspension can be compared then in this assay with an equal number of pathologic platelets from some diseased state Two determinations with this procedure should agree to within 10 per cent if platelets are active. A typical experiment with normal platelets can be followed in table 1.

TABLE 1

Reaction Time	Thrombin u /ml
10	160
25	470
40	965
60	1000

It is essential to find the lowest concentration of normal platelets required to convert all of the purified prothrombin to thrombin and so suitable dilution of the platelet suspension is carried out before the procedure is begun to ascertain that the activation of purified prothrombin is being carried out at a critical level Then if unknown platelets give a yield of thrombin below 1000 units, they have less platelet factor 3 activity than is normal The greater the deficiency the less will be the thrombin yield.

to 1,000,000 cu mm Just before assay the platelet suspension should be thawed rapidly and treated to sonic disintegration of 9 kc for 2 minutes

(4) *Calcium chloride* A solution of 0.01 M calcium chloride is prepared by dissolving the calcium chloride in 25 ml. of physiologic saline and then made up to 100 ml. volume by addition of imidazole buffer. Imidazole is the buffer to be used in all two-stage determinations.

(5) *Thrombin.* The conversion of purified prothrombin to thrombin in this test proceeds most rapidly if seeded with thrombin It is the autoprothrombin C component of the thrombin which is the active ingredient. Thrombin Topical of Parke, Davis & Co. can be used as purchased or purified further in the following manner according to Johnson, Caldwell and Priest As purchased it has a specific activity of up to 500 units per mg. tyrosine. Three vials are dissolved in 10 ml of 25 per cent sodium citrate and left at room temperature for 2 hours, converting biothrombin to citrate thrombin. The citrate thrombin can be precipitated by 37 per cent sodium citrate in a 25 ml. volume for 45 minutes, and then centrifuged at 1500 g for 20 minutes The resulting sediment is dissolved in 5 ml. distilled water and dialyzed against physiologic saline. This thrombin should have a specific activity of 10,000 to 20,000 units per mg. tyrosine

(6) *Thrombin diluent:* This solution is essentially the same as that used by Seegers as described in this volume on page 184. It is sometimes convenient to replace acacia with dextran according to Owen, Hurn and Mann. Dextran can be purchased from Abbott Laboratories, North Chicago, and is in the concentration of 6 per cent w/v in saline

Thrombin Diluent

Dextran (6%)	50 ml
Imidazole buffer (pH 7 2-7 4)	25 ml
$CaCl_2$ (0 70%)	50 ml
NaCl (0 85%)	25 ml

To 10 ml of this solution add 5 ml of physiologic saline

(7) *Fibrinogen* A standard 1 Gm per cent solution of fibrinogen such at that described by Seegers and Johnson (p. 228) may be used. An alternative is a 2 Gm per cent solution of bovine fibrinogen from Armour Pharmaceutical Co , Kankakee, Ill This concentration usually results in a fibrinogen solution containing about 1 2–1 4 Gm per cent clottable fibrinogen The solution should be stored in the deep freeze in aliquots of about 5 ml. and thawed when needed.

Steps in performance of the test Into a test tube place the following reagents in the order given.

centrifuge tube containing 1 ml. of 1 per cent Sequestrene solution Mix by inverting the tube. The donor blood is centrifuged at 40° C. for 15 minutes at 1000 r p m The upper two-thirds of the plasma is transferred to a clean siliconized test tube. This platelet-rich plasma must be standardized by count. A chamber platelet count of the separated platelet-rich plasma is done and if necessary the count is adjusted to approximately 200,000 per cu mm by dilution with platelet-poor plasma from the same donor (refer below). Then the plasma is refrigerated at 4°C. until ready for use. (2) *Blood collection from patient and controls for platelet-poor plasma:* Collect venous blood and immediately place in test-tube containing 1 per cent Sequestrene solution: proportion 1 part Sequestrene to 9 parts of blood. The blood is centrifuged at 4°C. for 30 minutes at 3000 rpm. The upper two-thirds of the platelet-poor plasma is transferred to a clean test tube. A known negative control is treated in the same way. (3) *Agglutination test·* Into a 75 × 8 mm. test tube, place 0.1 ml. of donor platelet-rich plasma, and 0 1 ml of the patient platelet-poor plasma. Prepare a tube in this manner for the control Each tube is stoppered and allowed to stand in a water bath at 37°C. for 60 minutes At the end of this period, the tubes are centrifuged at 500 rpm for 2 minutes; each tube is then shaken gently to resuspend the platelets. One drop of each mixture is placed on a siliconized glass slide and without pressure a cover slip is applied to each mount The preparations are read at high-dry magnification for the presence of platelet clumps

Interpretation of Results The presence of platelet clumps (8-10 platelets clumped in definite formation), and these clumps present throughout many microscopic fields, is a positive finding A homogeneous distribution of platelets is negative

B *Serum Method of Harrington, Minnich and Arimura*[2]

Principle After incubation for 24 hours at 37°C and inactivation at 56°C, sterile serum is mixed with platelet-rich plasma from a normal donor or with autologous platelet-rich plasma for the detection of iso- and auto-antibodies, respectively The mixture is incubated at 50°C, and the results read microscopically using depressed glass slides

Apparatus and Reagents (1) *Glassware* Except as otherwise noted, the glassware must be siliconized including syringes, aspirating and measuring pipettes, depressed type microslides; test tubes, 75× 10 and 125 × 16, and 15 ml centrifuge tubes (2) *Solutions and reagents* Platelet count diluting fluid, 0.13 M Sequestrene (disodium EDTA), and human ABO blood grouping sera. (3) *Equipment* A refrigerated 4°C centrifuge, a mechanical oscillating platform, water bath controlled at 37°C and 56°C.,

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11. Detection of Platelet Agglutinins

Described by P. GEISLER and M. EICHMAN

Object: Detection of agglutinating platelet antibodies in the plasma or serum of thrombocytopenic patients

A. *Rapid Plasma Method of Stefanini and Dameshek*[1]

Principle Platelet-poor plasma from the patient is mixed with platelet-rich plasma from normal donors for detection of isoantibodies, or with the patient's own platelet-rich plasma for detection of autoantibodies. Results are read microscopically as a slide agglutination test.

Apparatus and Reagents. (1) *Glassware.* the glassware is coated with silicone which includes syringes, collecting tube (15 ml graduated centrifuge test tube is convenient), test tubes 75 × 8 mm, aspirating and serologic pipettes, micro glass slides, cover slips 22 × 22mm (2) *Reagents:* Sequestrene (disodium EDTA) recrystallized (Geigy Chemical Corp, Ardsley, N Y), 1 per cent aqueous solution, and 1 per cent Sequestrene in 0.7 per cent sodium chloride, human ABO blood grouping sera (3) *Apparatus:* a refrigerated centrifuge (4°C.) with controllable speed is of great help in obtaining reproducible and reliable results, a blood counting chamber and blood diluting pipettes, microscope, water-bath at 37°C and test tube stoppers protected with Parafilm (Marathon Corp., Menasha, Wis.).

Procedure: (1) *Blood collection from platelet donors for platelet-rich plasma* Group 0 or a group compatible with the patient's group is used. With clean venepuncture collect 9 ml of blood and immediately place into a

and positive for platelet agglutinins should be included as controls (4) In our studies, we consider a panel of a minimum of four platelet donors If it is not possible to obtain this desired number of platelet donors on one day, the patient plasma or serum is stored in small aliquots at −20°C. It has been recommended that specimens not be thawed and refrozen; thus the small aliquot storage. (5) In reporting our findings to clinicians we do not qualify the degree of agglutination of the test microscope readings. However, we have established criteria for "positive" reporting, i e., the presence of clumped platelets, eight to ten en masse, throughout many fields.

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microscope, blood counting chamber, blood diluting pipettes, and test tube stoppers protected with Parafilm or aluminum foil.

Procedure. From a group O blood donor or one that is compatible with the patient's group, applying only moderate tourniquet pressure, collect 10 ml of venous blood, and immediately transfer the blood to a test tube containing 0 3 m. of disodium Sequestrene, 0.13 M. The tube is covered with foil or Parafilm and inverted to mix. The blood is centrifuged at 1000 rpm for 15 minutes, preferably in a refrigerated centrifuge at 4° C. Separate the plasma, and do a platelet chamber count The platelet-rich plasma count should be 3–400,000 per cu mm If necessary, the count is adjusted by recentrifugation or with the addition of platelet-poor plasma from the donor (see below for preparation of platelet-poor plasma). This standardized platelet-rich plasma is refrigerated until ready for use The donor *plasma should not be prepared until ready for use, i.e, 24 hours after* making the blood collection from the patient.

The patient's blood is collected aseptically in a sterile, unsiliconized glass tube, using an 18 gauge needle. The unclotted blood is incubated at 37°C. for 24 hours. The blood is centrifuged at 3000 rpm for 15 minutes, and the sterile serum is transferred to a second test tube and inactivated at 56°C. for 30 minutes One-tenth ml of the 0 13 M disodium Sequestrene solution is added to 2 ml of the inactivated serum, and 0.3 ml. of this treated serum is added to 0.1 ml of the donor platelet-rich plasma in a 75 × 10 mm. test tube. The tube is shaken gently and incubated overnight at 50°C. The following morning (about 18 hours), each tube is shaken *gently to resuspend the platelets, and half of the contents of each tube is poured into one of the depressions of the micro-slide* The slides are kept in a moist chamber for 30 minutes, then agitated on a mechanical oscillating platform or by hand at 40 rpm for 5 minutes. The tests are read at 100 × magnification, and observed for platelet clumping.

Interpretation of Results The degree of platelet agglutination or clumping may be graded 0 to 4+ In occasional instances, lysis instead of agglutination of platelets may be noted For purposes of titration, serial dilutions of the patient serum may be made using group AB serum or serum from the platelet donor.

Precautions and Sources of Errors. (1) In our experience,[3] the platelet-rich suspensions should be used on the day of preparation and preferably, as recommended by Stefanini, within 4 hours of preparation; otherwise, we have noted confusing morphologic changes in the platelets when microscopically examined (2) Preferably, the platelet donors should be screened for platelet agglutinins, and also should have no history of blood-therapy and/or pregnancy (3) Whenever possible, sera known to be negative

and positive for platelet agglutinins should be included as controls. (4) In our studies, we consider a panel of a minimum of four platelet donors. If it is not possible to obtain this desired number of platelet donors on one day, the patient plasma or serum is stored in small aliquots at −20°C. It has been recommended that specimens not be thawed and refrozen; thus the small aliquot storage. (5) In reporting our findings to clinicians we do not qualify the degree of agglutination of the test microscope readings. However, we have established criteria for "positive" reporting, i.e., the presence of clumped platelets, eight to ten en masse, throughout many fields

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[3] Eichman, M F Antibodies for platelets in clinical medicine Am J Med Technol. *28* 301-308, 1962

microscope, blood counting chamber, blood diluting pipettes, and test tube stoppers protected with Parafilm or aluminum foil

Procedure: From a group 0 blood donor or one that is compatible with the patient's group, applying only moderate tourniquet pressure, collect 10 ml. of venous blood, and immediately transfer the blood to a test tube containing 0.3 m. of disodium Sequestrene, 0.13 M. The tube is covered with foil or Parafilm and inverted to mix. The blood is centrifuged at 1000 rpm for 15 minutes, preferably in a refrigerated centrifuge at 4° C. Separate the plasma, and do a platelet chamber count. The platelet-rich plasma count should be 3–400,000 per cu. mm If necessary, the count is adjusted by recentrifugation or with the addition of platelet-poor plasma from the donor (see below for preparation of platelet-poor plasma). This standardized platelet-rich plasma is refrigerated until ready for use The donor plasma should not be prepared until ready for use, i.e., 24 hours after making the blood collection from the patient

The patient's blood is collected aseptically in a sterile, unsiliconized glass tube, using an 18 gauge needle The unclotted blood is incubated at 37°C. for 24 hours. The blood is centrifuged at 3000 rpm for 15 minutes, and the sterile serum is transferred to a second test tube and inactivated at 56°C for 30 minutes. One-tenth ml. of the 0.13 M disodium Sequestrene solution is added to 2 ml of the inactivated serum, and 0.3 ml. of this treated serum is added to 0 1 ml of the donor platelet-rich plasma in a 75 × 10 mm test tube The tube is shaken gently and incubated overnight at 50°C The following morning (about 18 hours), each tube is shaken gently to resuspend the platelets, and half of the contents of each tube is poured into one of the depressions of the micro-slide. The slides are kept in a moist chamber for 30 minutes, then agitated on a mechanical oscillating platform or by hand at 40 rpm for 5 minutes The tests are read at 100 × magnification, and observed for platelet clumping

Interpretation of Results The degree of platelet agglutination or clumping may be graded 0 to 4+. In occasional instances, lysis instead of agglutination of platelets may be noted. For purposes of titration, serial dilutions of the patient serum may be made using group AB serum or serum from the platelet donor

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plasma preparation is mixed with an equal volume of ethyl ether and shaken for three minutes. The ether extraction is repeated three times The material is stored in the deep freeze.

Thrombin Activity: Determine according to the method described on page 181.

Serum: Blood is permitted to clot at room temperature; after two hours it is centrifuged at 1500 g for 30'.

Procedure· Into a silicone-coated 10 ml. test tube are placed: 1 ml. of the unknown plasma or serum, 0 5 ml $CaCl_2$ (0.153 M) in imidazole buffer, 0 5 ml. platelet extract, 1 ml purified prothrombin. Aliquots are removed at timed intervals with a silicone coated pipette The reacting mixture is suitably diluted to have a final concentration, when added to fibrinogen, of approximately 1 unit per ml.

Calculation· The total units of thrombin formed are plotted on graph paper against the time the aliquot is removed in minutes Normal plasma produces a full thrombin yield in 30 minutes after a few minutes latent period

Precautions and Sources of Error. The concentration of prothrombin in the mixture must be sufficiently high so that the prothrombin content of the added plasma may be ignored due to the high dilution of the plasma in the test system.

The reacting mixture must be adequately diluted to yield a thrombin concentration low enough to be accurately measured (about 1 unit per ml). The plasma must be shaken with the ether for at least 1½ minutes to destroy antithrombin.

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CHAPTER VI

PLASMA THROMBOPLASTIN AND PRECURSORS

1. *Demonstration of Platelet Cofactor Activity in Plasma (Method of Johnson)*

Described by R. R. HOLBURN*

Object of Method: Demonstration of the presence of a plasma globulin which, in conjunction with a platelet extract, activates purified prothrombin to thrombin Measurement of thromboplastin-like activity arising from the interaction of platelet extract and the plasma globulin.

Principle: If a mixture of platelet extract, calcium and purified prothrombin is made, and a specially treated plasma is added, the amount of thrombin formed will depend on the amount of platelet cofactor available in the plasma. The activity formed can substitute for tissue thromboplastin.

Apparatus and Reagents Purified prothrombin· Prepared according to the method described elsewhere (see page 174). Solution made to contain about 3,000 U/ml

Platelet extract Platelets are obtained by differential centrifugation and washed three times with 0 85 per cent NaCl One part of the packed platelets is mixed with 9 parts of 0 85 per cent NaCl and frozen Then 10 ml of the frozen suspension is thawed and centrifuged (1500 g for 30') in the cold The sediment is recovered, washed once in 0.85 per cent NaCl and resuspended in 2 ml of 0 85 per cent NaCl.

Plasma samples: A clean venepuncture, using a siliconed syringe, is made and the first syringe replaced with another, after 2 ml of blood is drawn and discarded Then 10 ml of blood are placed immediately in a silicone coated centrifuge tube containing 1 0 ml of 0 112 M potassium oxalate The two are mixed and the plasma is removed after centrifugation (1500 g for 30' at 8°C) Plasma is defibrinated by the addition of an equal volume of thrombin containing 20 U /ml. To destroy antithrombin, the

* From American Journal of Clinical Pathology 23 875, 19..

euglobulin was prepared as the abscissa values. If the euglobulin of the unknown plasma is normal in amount and function, the suspensions prepared from the more dilute plasmas will gain in clot-promoting activity until equal to that of the normal euglobulin suspension The degree of dilution required to equalize the activity of the 2 euglobulins is an approximate measure of the qualitative defect of the unknown plasma.

Values Obtained: Normal plasma euglobulin solutions when prepared from plasma diluted 1:1000 display a two-fold increase in clot-accelerating activity over that prepared from plasma diluted 1:10 (fig. 1). Further dilution of the plasma used to prepare the euglobulin solution produces no greater increase in clot-promoting power. Hemophilia A plasma euglobulin solutions prepared from a 1:10 plasma dilution do not accelerate the clotting of normal plasma to any great extent and are powerless when tested on a substrate of their own plasma. However, as the plasma used in the preparation of the solution becomes increasingly more dilute, the euglobulin from hemophilic plasma gain markedly in clot-promoting power until they function as well as the normal euglobulin prepared from an equivalent

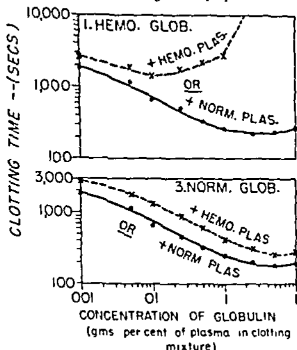


FIG 1 Effect of dilution on the clot accelerating activity of normal and hemophilic euglobulin solutions tested on normal and hemophilic plasma

2. Estimation of the Clot-Promoting Power of Plasma Euglobulin Prepared by the Method of Progressive Dilutions

L. M. TOCANTINS, R. R. HOLBURN and R. T. CARROLL

Object of the Method: A test for the presence and function of clot promoting euglobulins in normal and abnormal plasmas

Principle. If the clot promoting euglobulin is present in plasma in normal amount and function, it will gain in activity as it is prepared from increasingly dilute plasma.

Apparatus and Reagents: Platelet-poor plasma. Use of silicone surfaces throughout the processing and collection is necessary. Blood from a clean puncture of a turgid vein using a #18 needle is drawn into a syringe containing 0.2 ml. 19 per cent sodium citrate per 10 ml blood and the mixture well mixed by inversion. The citrated blood is allowed to run down the wall of a silicone coated tube by applying gentle pressure to the plunger of the syringe The plasma is separated from the cells by centrifuging at 2400 g for 50 minutes at 4°C and removed by a silicone dropper pipette avoiding the agitation of the cellular elements.

Euglobulin solutions: Four samples of the platelet-poor plasma from the normal and the unknown blood are each diluted with distilled water 1:10, 1:100, 1 1000 and 1:10,000 Euglobulins are precipitated by the dropwise addition of 1 per cent acetic acid to pH 6 0 After each solution is allowed to stand in the refrigerator for 1 hour, it is centrifuged for 30 minutes at 2400 g at 4°C The supernatant is discarded and the sediment redissolved in a volume of 0 85 per cent NaCl solution equal to the original volume of plasma used Alternately the sediments are dried in the dessicator overnight, weighed and 0 4 per cent solutions in 0 85 per cent NaCl prepared for testing. The pH is adjusted to 7 4 with a drop of imidazole buffer. The solutions are tested immediately and should stand in ice before the test period since they will clot if allowed to stand too long

Steps in Procedure The testing is carried out in a 37°C water bath and in silicone tubes Only the pipettes used for plasma need, however, to be siliconized To 0 5 ml of stable platelet-poor plasma, 0 1 ml euglobulin suspension, 0 05 ml. 0.2 M $CaCl_2$, are added and the stopwatch is started at the addition of the calcium chloride. Each euglobulin suspension is tested on a substrate of its own plasma as well as normal plasma

Calculation: The data obtained is plotted on log-log graph paper with clotting times as the ordinate and the plasma concentration from which the

in 10 minutes (fig. 2). The difference between normal and Hemophilia A plasmas in their response to Hemophilia A euglobulin can be eliminated by appropriate dilution of the plasmas to about 5 per cent concentration. Euglobulin solutions prepared from Hemophilia A plasma at 1 per cent or less concentration accelerate the coagulation of Hemophilia A blood in vitro and in vivo to an essentially similar degree as when the euglobulin suspension originates from normal plasma at 10 per cent concentration.

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3. Thromboplastin Generation Test of Biggs and Douglas[1]

R. L MacMILLAN

Object of Test. To measure the formation of thromboplastin from intrinsic factors of the blood

Principle Biggs and Douglas[1] showed that normal blood contains factors which in vitro generate a powerful thromboplastic activity capable of clotting platelet-poor plasma in 9 to 10 seconds Four components are required in the thromboplastin generation mixture Platelets contribute a lipid fraction The second component required for thromboplastin generation is factor VIII or antihemophilic factor Plasma adsorbed by agents such as aluminum hydroxide or barium sulphate provide this factor. The third component is factor IX or plasma thromboplastin component (P.T.C. or Christmas factor) Normal serum contains this factor and is a convenient source Finally, calcium must be added to the mixture to activate thromboplastin generation. As well as these factors, it is now realized that factor V

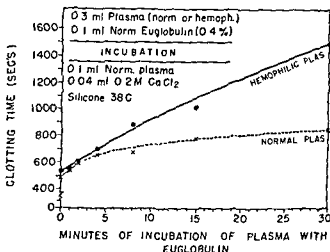


Fig 2 Diminished activity of normal euglobulin after incubating with normal or hemophilic plasma

dilution, whether tested on a substrate of normal or hemophilic plasma. The amount of dilution necesary to equalize the activity of the hemophilic euglobulin suspension with that of the normal depends on the severity of the defect

Solutions prepared from plasma which has a quantitative deficiency in the clot promoting euglobulin (e.g hemophilia B plasma) cannot, regardless of amount of dilution, equal normal euglobulin suspensions in clot-promoting power whether tested on a substrate of its own plasma or normal plasma

Precautions and Sources of Error Care must be taken to recover all the precipitated euglobulin from the highly diluted specimens. The weight of the dried euglobulin precipitate, after correction for salt content, will serve as a check for complete recovery Incubation of the euglobulin solution with plasma before recalcification results in the loss of activity that may be complete in 30 minutes Therefore, recalcification should follow at once after addition of the euglobulin solution to the plasma

Discussion Hemophilia A euglobulin prepared from 10 per cent plasma concentration has little clot-promoting activity while normal euglobulin prepared from the same concentration has a strong activity As more dilute plasma is used for the preparation of the euglobulins, the activity of the hemophilic euglobulin gradually increases and eventually equals that of the normal When a highly active normal euglobulin is incubated with citrated normal plasma before recalcification, much of its activity is lost within 30 minutes, when incubated with Hemophilia A plasma, it loses all activity

in 10 minutes (fig 2). The difference between normal and Hemophilia A plasmas in their response to Hemophilia A euglobulin can be eliminated by appropriate dilution of the plasmas to about 5 per cent concentration Euglobulin solutions prepared from Hemophilia A plasma at 1 per cent or less concentration accelerate the coagulation of Hemophilia A blood in vitro and in vivo to an essentially similar degree as when the euglobulin suspension originates from normal plasma at 10 per cent concentration.

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3. Thromboplastin Generation Test of Biggs and Douglas[1]

R. L MacMILLAN

Object of Test To measure the formation of thromboplastin from intrinsic factors of the blood

Principle Biggs and Douglas[1] showed that normal blood contains factors which in vitro generate a powerful thromboplastic activity capable of clotting platelet-poor plasma in 9 to 10 seconds. Four components are required in the thromboplastin generation mixture Platelets contribute a lipid fraction The second component required for thromboplastin generation is factor VIII or antihemophilic factor Plasma adsorbed by agents such as aluminum hydroxide or barium sulphate provide this factor The third component is factor IX or plasma thromboplastin component (P T.C. or Christmas factor) Normal serum contains this factor and is a convenient source Finally, calcium must be added to the mixture to activate thromboplastin generation As well as these factors, it is now realized that factor V

of alumina plasma, factor X of serum and the contact factors XI and XII are necessary for normal thromboplastin generation The thromboplastin generation mixture consists of platelets or platelet substitute, adsorbed plasma, serum and calcium. After a normal system has been studied, a comparison may be made with the generation that occurs in a mixture containing one or more components prepared from the blood of subjects under investigation for clotting defects.

Reagents and Apparatus: (1) *Collection of blood.* Venous blood is obtained by venepuncture. Nine parts of blood are mixed with 1 part of 3 8 per cent sodium citrate solution

(2) *Platelets* Freshly collected blood is centrifuged at 1000 rpm for 10 minutes. The red and white cells are deposited at the bottom of the test tube while the platelets remain in suspension in the supernatant plasma. The platelet-rich plasma is pipetted off and spun in a high speed centrifuge at 15,000 rpm for 5 minutes or in an ordinary centrifuge at 3000 rpm for 20 minutes The platelets form a sticky plug at the bottom of the tube Supernatant platelet-poor plasma is poured off and kept for use later as substrate The platelet plug is broken up with a wooden applicator stick and is washed with 5 ml. of physiologic saline The washed platelets are centrifuged again at high speed and the washing is repeated twice. Finally, the platelets are resuspended in physiologic saline to a volume equal to one-third of the original plasma If the function of platelets is to be studied in the system it is important to prevent change to the platelet membrane as far as possible, the glassware is carefully coated with silicone and the washing and mixing of platelets carried out as gently as possible

(a) *Platelet substitute* Bell and Alton[2] have described a chloroform extract of brain that is a satisfactory substitute for platelets in the thromboplastin generation mixture More highly purified phospholipid fractions of brain prepared by the method of Folch have also been studied In incubation mixtures these purified extracts have given contradictory results. Fraction IV of Folch appears to be the most active although it is impure, containing components from both fractions III and V Soya bean extract can be used as a platelet substitute and is mixed with physiologic saline or buffered saline The dispersion of the material is aided by freezing and thawing A dilution of 1 in 100 with physiologic saline is generally satisfactory The activity of soya bean suspension decreases on storage in the frozen state making it necessary to prepare a fresh mixture each week

(3) *Factor VIII (antihemophilic globulin)* Aluminum hydroxide is prepared by the method of Bertho and Grassman as described by Biggs and Macfarlane.[3] One-tenth ml of the alumina gel is added to 1 ml of

citrated plasma After incubation at 37°C. for 2 minutes, the mixture is centrifuged and the supernatant plasma is diluted 1:5 with physiologic saline. Aluminum hydroxide gel prepared by British drug houses may also be used. After adsorption, the one-stage prothrombin time of the plasma should be greater than 60 seconds.

(4) *Factor IX (P.T.C. or Christmas factor)* Normal blood is allowed to clot and the serum separated by centrifugation. The serum is allowed to stand at room temperature overnight to allow complete disappearance of thrombin, prothrombin and thromboplastin. A 1 in 10 dilution with physiologic saline is used in the test.

(5) *M/40 calcium chloride solution Performance of test.* During the test the reagents are kept in a bath of melting ice to decrease loss of activity of the clotting factors Before preparing an incubation mixture, 0.2 ml. aliquots of platelet-poor plasma are placed in six small uniform test tubes in a water bath at 37°C. The thromboplastin generation mixture is prepared by placing in a test tube 0.2 ml. of platelet suspension or platelet substitute, 0.2 ml. of 1:5 dilution of alumina plasma and 0.2 ml of 1:10 dilution of normal serum The incubation mixture is placed in the water bath at 37°C. and 0.2 ml of M/40 calcium chloride solution is added At this point a stop watch is started and, at 2-minute intervals, samples of 0.1 ml are removed by means of a Pasteur pipette held in the right hand. Another pipette containing 0.2 ml of M/40 calcium chloride solution is held in the left hand Both pipettes are discharged simultaneously into one of the small tubes containing 0.2 ml of platelet-poor plasma, previously placed in a water bath. The clotting time of the plasma is measured by a second stop watch The shortest clotting time usually occurs at the end of 6 minutes and should be between 9 and 11 seconds. After this the clotting time becomes longer, probably due to destruction of thromboplastin by antithromboplastin During incubation a small clot forms in the mixture due to fibrinogen introduced in the alumina plasma This clot is wrung out on the Pasteur pipette and discarded. Next, alumina plasma or serum prepared from patients under investigation for clotting defects may be substituted for normal components in the generation mixture If this results in the thromboplastin generation becoming impaired, it may be assumed that the component substituted lacks a factor necessary for normal thromboplastin formation For example, if thromboplastin generation is poor where alumina plasma from a patient is substituted for normal plasma, it is probable that the plasma lacks factor VIII and that the patient has classical hemophilia If defective thromboplastin generation occurs when serum is substituted in an otherwise normal system, the diagnosis is likely factor IX deficiency (P T C deficiency or Christmas disease) (table 1)

TABLE 1. *Thromboplastin Generation in (1) Normal Mixture, (2) Mixture Containing Serum from Patient Stephen Christmas (Factor IX Deficiency)*

Source of serum in the thromboplastin generation mixture	Incubation time in minutes					
	2	4	6	8	10	12
	Clotting time in seconds					
Normal	45	16	10	10	12	15
Patient—Stephen Christmas (factor IX, P.T.C. deficiency)	62	35	24	22	26	30

In plasma thromboplastin antecedent (P.T.A.) deficiency, thromboplastin generation is abnormal when the mixture contains both the patient's alumina plasma and serum The presence of either normal plasma or normal serum results in a varying degree of improvement in thromboplastin generation

Calculations: The thromboplastin generation test may be used to determine the level of factor VIII in the blood of hemophilic patients receiving blood or plasma transfusions To perform this assay it is necessary to prepare a series of dilutions of normal adsorbed plasma using an adsorbed plasma from a patient with severe classical hemophilia (factor VIII) deficiency as diluent As an alternative to hemophilic plasma, citrate-saline may be used as diluent provided factor V is added to the mixture to maintain a constant level. The shortest clotting time for each dilution is plotted against the dilution of normal plasma tested on double logarithmic paper. The points should lie on a straight line Dilutions of the patient's adsorbed plasma are next tested in the same way and the results are plotted on a line parallel to the normal curve. From this graph the percentage of factor VIII in the patient may be calculated[3] It is interesting to note that the method using hemophilic plasma as diluent gives different results from the citrate-saline method on the same blood sample This discrepancy indicates that the assay of factor VIII by the thromboplastin generation test is semi-quantitative

Normal Range of Values Considerable variation is noted in the thromboplastin generation test from day to day in the same laboratory. It is necessary each day at the outset to establish a normal generation system. Fortunately, the difference between normal and abnormal mixtures is usually large enough to make the test useful as a diagnostic procedure

Precautions and Sources of Error. Several hours are required to prepare for and to carry out thromboplastin generation tests During this time dilutions of serum and alumina plasma must be kept in a bath of melted ice to avoid loss of activity Platelet substitutes may prove troublesome.

Brain extracts have an optimum dilution that may change unaccountably during storage. Soya bean phospholipid is stable in the powdered state but in solution loses activity after a week's storage even when kept frozen. If the shortest clotting time with the normal mixture is longer than 11 seconds, new normal components should be prepared and the test repeated.

It is important to realize that barium sulphate adsorbs clotting factors from oxalated plasma very well but not from citrated plasma. With citrated plasma, aluminum hydroxide must be used as the adsorbing agent. In addition, there is considerable difference in the ability of different aluminum hydroxide preparations to adsorb clotting factors such as prothrombin, and factors VII and IX. Adsorbed plasma should have a one-stage prothrombin time of approximately 60 seconds to be satisfactory in the thromboplastin generation mixture. If the prothrombin time is excessively prolonged, the system will be inactive. Factor VIII may be adsorbed under certain conditions from the plasma by aluminum hydroxide

Fresh serum is unsuitable because it contains thrombin The length of time that serum should be kept before being used in the test is not settled. There is evidence that serum loses thrombin activity after 3 hours at 37°C. Seaman and Karlsen[4] have found thromboplastin generation with soya bean phosphatide less variable from day to day if the serum is incubated for only 1 hour at 20°C Longer incubation at higher temperatures results in loss of activity Where a large number of tests are to be carried out, pooled lyophilized serum may be used Dr. J. F. Mustard (personal communication) found that serum from menstruating females was inactive in the test

When the thromboplastin generation test is used to study plasma thromboplastin antecedent (P T A) deficiency, the results are often difficult to interpret. Minor abnormalities in factors VIII and IX appear to be present when only a single component of the patient's blood is included in the mixture, suggesting the patient may have a mild lack of either factor VIII or IX

In interpreting the results of the thromboplastin generation test it is important to remember the warning by Dr Rosemary Biggs,[5] "The method assumes specifically that the minimum clotting time by this technique is related to factor VIII (antihemophilic globulin) or factor IX (Christmas factor) concentration and that no other factor not already thought of and taken into account will have any effect Now this of course is a very arrogant assumption and very likely untrue " Minor differences in technic in performing the test are not important What is important is that each laboratory standardize their methods for the preparation of the reagents and the performance of the test If this is done carefully the test becomes reproducible from day to day and is very useful as a diagnostic procedure

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4. Rapid Method for Screening Disorders of Thromboplastin Generation (Method of Hicks and Pitney[1])

Described by R. R. HOLBURN and M. DeSIPIN

Object of Test. A rapid screening test for disorders of thromboplastin generation.

Principle Diluted plasma is recalcified in the presence of platelets or a platelet substitute and the resulting thromboplastic activity is measured by adding the reacting mixture to normal plasma

Preparation of Reagents. Collection of blood. Forty-nine parts of venous blood are immediately mixed with one part of 19 per cent sodium citrate and the plasma separated by centrifugation at 3000 r p.m for 10 minutes at 4°C The plasma is stored at 4°C. for testing the same day *Platelet substitute* A chloroform extract of acetone-dried human brain is prepared as described by Bell and Alton [2] The dried material is suspended in physiologic salt solution and stored at −20°C Saline dilutions of this suspension are tested with normal plasma and the dilution giving the shortest clotting time after recalcification of the substrate plasma is used The stock suspension maintains its potency for several months at −20°C. The diluted suspension is made fresh daily

Substrate plasma. Normal plasma is separated from venous blood collected and processed in the same manner as above Pooled or individual samples of plasma are satisfactory *Calcium chloride* 0.02 M $CaCl_2$ is used.

Performance of the Test: The plasma to be tested is diluted with buffered saline (0 9 per cent NaCl solution, buffered with 0 05 M imidazole buffer), pH 7.3 to 7 4, one part plasma plus nine parts diluent. In a glass tube, 70 x 12 mm., in a 37°C. water bath, 0 5 ml. diluted plasma is mixed with 0 5 ml. platelet substitute. After exactly 1 minute, 0 5 ml of 0 02 M $CaCl_2$ solution is added rapidly and the first stopwatch started. At 1-minute intervals, successive 0 1 ml. samples are removed from the mixture and added to a series of test tubes, containing 0 1 ml. substrate plasma previously placed in the water bath. Immediately after the addition of the incubation mixture, 0.1 ml. 0 02 M $CaCl_2$ solution is added and the clotting time measured with a second stop watch. The clot which forms in the incubation mixture is easily removed by a wooden applicator stick.

Normal Range of Values: In tests on plasma separated from 106 normal subjects, minimum substrate clotting times of 11 seconds or less are reached in 3 to 5 minute of incubation. Patients with hemophilia A or B have abnormal generation curves. Plasma from these patients may result in minimum substrate clotting times longer than those of normal individuals, or in a slower generation.

Precautions and Sources of Error. The test is non-specific and cannot distinguish between hemophilia A and B It is believed to be insensitive to factor VII deficiency The plasma dilution should be tested as soon as it is made because it will gain in reactivity as it stands. This phenomenon is especially found in some abnormal plasmas The effect is most marked in glass tubes but also occurs in siliconized test tubes even when stored at the temperature of melting ice. The period of prewarming of the reaction mixture before recalcification must be the same for the control and test plasmas

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5. Estimation of Thromboplastin Generation Accelerator (TGA) in Human Plasma*

C. A. OWEN, JR., J. H. THOMPSON, JR. and J. A. SPITTELL, JR.

Object of the Test: In certain patients with arterial thrombosis or idiopathic recurrent thrombophlebitis, hypercoagulability is manifested in the thromboplastin generation test This has been explained as being caused by an excess of a normal coagulation factor: thromboplastin generation accelerator or TGA.[1] The object of this test is to recognize an accelerated generation of thromboplastin and to distinguish TGA from factor VIII (AHG), which is increased postoperatively and in patients with extensive malignant disease.

Principle. TGA, when present in excess, accelerates the generation of thromboplastin from barium sulfate-adsorbed plasma but not from serum since the factor apparently disappears during clotting. The acceleration is seen most clearly when the reaction that generates thromboplastin is retarded by increasing the dilution of the plasmatic reagent.[2] If acceleration is detected, the plasma is treated with aluminum hydroxide which removes TGA but not AHG from the plasma

Reagents and Apparatus These are similar to those needed for the thromboplastin-generation test (TGT)[3]

(1) Control and patient's oxalated plasma (from nine volumes of blood plus one volume of 0 1 M sodium oxalate)

(2) Control serum (clotted blood is allowed to stand in glass for 24 hours at room temperature; the serum is removed after brief centrifugation).

(3) Soybean phosphatide is used as a platelet substitute (Inosithin, Associated Concentrates Co, L I, N Y). A 5 per cent emulsion of Inosithin is made by grinding the Inosithin with a small amount of buffered saline until a smooth paste is obtained; the remainder of the buffered saline then is added with continuous grinding The emulsion is put into 15 ml tubes, 0 05 ml per tube; these tubes are kept at −20°C until needed, but not longer than 1 month For use, a tube is thawed and 9 95 ml. of buffered saline is added, making a final concentration of Inosithin of 0 025 per cent

* This investigation was supported in part by Research Grant H-5008, from the National Institutes of Health, U.S P.H S.

(4) Buffered saline consists of one volume of imidazole buffer, pH 7.2 to 7.3, plus two volumes of physiologic saline. It is kept in a refrigerator at 4°C.

(5) Decalcified substrate plasma: about 25 ml. of normal whole blood is mixed, by four or five inversions, with Dowex-50W resin (H^+, X12, 50–100 mesh; Microchemical Specialities Co., Berkely, Calif.) until the resin is uniformly dispersed The resin is prepared by putting about 1 Gm. into a 50 ml. tube which then is filled with 5 per cent aqueous sodium chloride. After thorough shaking, the resin is allowed to sediment and the fluid is decanted. The resin is treated this way 2 more times, then 3 times with physiologic saline Additional washes are necessary if the sixth supernatant is still acid to litmus Approximately a dozen tubes of resin are processed at once and the tubes are kept at room temperature, filled with saline, until ready for use. The saline is discarded just before the blood is added After mixing the blood with the resin, it is centrifuged for 15 minutes at 2000 rpm in an International Centrifuge No. 2, preferably refrigerated at 5°C. The plasma is pipetted off in 0.1 ml portions into disposable glass tubes (10 x 75 mm., 3 ml., Corning Glass Works, Corning, N. Y.) as needed for the day's tests; the tubes are kept in ice water. The rest of the plasma may be stored frozen, for at least 2 weeks, for later use.

(6) M/40 calcium chloride in water.

(7)) Barium sulfate powder (J T Baker Chemical Co , Phillipsburg, N. J.).

(8) Aluminum hydroxide suspension (about 4 per cent as aluminum oxide· Amphojel "without flavor," Wyeth Laboratories, Inc., Philadelphia, Pa.)

(9) Centrifuges, 37°C water baths, pans for holding metal test tube racks and ice cubes, pipettes, foot-pedal controlled timers

Steps in the Performance of the Test· (1) About 1 ml. each of the control and of the patient's oxalated plasma is mixed with about 50 mg. of barium sulfate, a stirring rod is used initially The mixtures are allowed to stand 5 minutes with occasional inversion, and then are centrifuged for 5 minutes at 2000 rpm in a small clinical centrifuge.

(2) One ml each of control and patient's oxalated plasma is mixed with 0 1 ml of aluminum hydroxide suspension The tubes are left for 1 hour at room temperature, and are inverted as often as necessary to prevent settling of the aluminum hydroxide The adsorbed plasmas are collected after 10 minutes of centrifugation at 2000 rpm in the clinical centrifuge.

(3) Just before the test is performed, each adsorbed plasma is diluted 25-fold or 50-fold with buffered saline (0 1 ml of plasma plus 2.4 or 4 9 ml of buffered saline), these tubes are kept in ice water

(4) The serum (only normal serum is used) is diluted 10-fold with buffered saline (0.1 ml. of serum plus 0.9 ml. of buffered saline).

(5) The thromboplastin generation test (TGT) requires a set of five tubes (disposable 3 ml. glass tubes), and begins with the preparation of the incubating mixture. The first tube contains 0.4 ml. of 1:25 diluted barium sulfate-adsorbed control plasma, 0.4 ml. of the diluted serum, 0.4 ml. of Inosithin suspension, and 0.4 ml. of the solution of calcium chloride, all added in the order given. The tube is inverted, a stop watch is started, and the tube is put into a 37°C. water bath. The other four tubes are set up in the same manner (each being prepared just before use), except that the second tube contains the patient's plasma diluted 1:25; the third, the control plasma diluted 1:50; the fourth, the patient's plasma diluted 1:50; and the fifth is a duplicate of the control plasma diluted 1:25 as a check on possible deterioration of reagents. In each of these four, the serum, Inosithin, and calcium are the same as in the first tube.

(6) As the mixture in (5) incubates, 0.1 ml. aliquots are removed at intervals and are pipetted into the tubes containing 0.1 ml. of substrate ("Dowex plasma"); these were put into a 37°C. water bath just before the test. The clot times are determined at 37° C., just as for determination of prothrombin time. Usual times for sampling are 1, 3, 5, and 7 minutes, and then each minute as long as needed. There is enough incubating mixture (1.6 ml.) to do 14 or 15 tests.

(7) When two or three consecutive clot times are practically the same, the TGT end point is reached, and an aliquot of the incubating mixture is diluted with an equal volume of buffered saline (50 per cent), and another with two volumes of buffered saline (33 per cent). Clot times now are determined with 0.1 ml. portions of these diluted specimens added to the substrate plasma. This furnishes a three-point curve (100, 50, and 33 per cent) for approximate quantitation of the generation of thromboplastin (the shortest clot time of undiluted incubating mixture = 100 per cent).

(8) Steps (5) to (7) are repeated exactly except that the aluminum hydroxide-treated plasmas are substituted for those adsorbed with barium sulfate. This step is omitted if the generation of thromboplastin was normal in step (6).

Manner of Expression of Results. The rate of generation of thromboplastin and the yield are estimated for the patient's plasma adsorbed with barium sulfate and with aluminum hydroxide at both the 1:25 and 1:50 dilutions, by using the controls as the standards in each case. Acceleration, if any, is expressed simply as "slight," "moderate," or "marked" based on the estimation of both the rates and yields. The result is qualified as "TGA" or "AHG" according to whether the acceleration disappears after adsorption with aluminum hydroxide (TGA) or not (AHG).

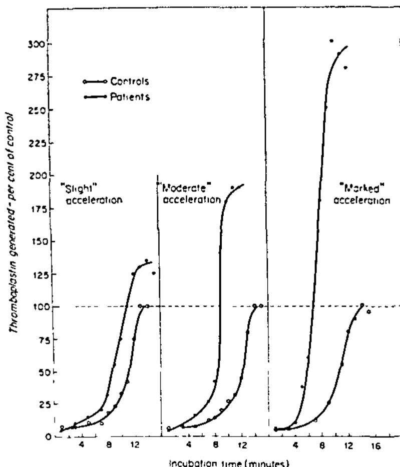


FIG 1 —Examples of acceleration in plasmas from three different patients

Calculations: These are best shown by actual results. Figure 1 illustrates examples of acceleration in plasmas, from three different patients, classified as "slight," "moderate," and "marked" In all three, the yields are increased (130, 190, and about 300 per cent of control). The half-time (time in minutes until half the ultimate yield is reached) also are increased, for the controls these ranged from 11 to 12 minutes, and for the patients, 10, 8½, and 7½ minutes. In other samples of plasma exhibiting acceleration, the primary abnormality may be in rate r' .ne or in yield alone

Table 1 shows the results of a test with a plasma in which "slight to moderate" acceleration is attributable to an excess of TGA (corrected by

adsorption with aluminum hydroxide). Table 2 shows the results with a plasma with "moderate" acceleration; there was no correction after adsorption with aluminum hydroxide. In this test, therefore, the acceleration was ascribable to excessive AHG; excessive factor V activity was excluded on other grounds.

Normal Range of Values: At 1:25 dilution, barium sulfate-adsorbed plasma normally yields end points of 8 to 11 seconds after 7 to 11 minutes

TABLE 1 *Results in Thromboplastin Generation Test on Plasma Containing Excessive TGA*

	Clot Times (seconds)									
	Plasma* adsorbed with $BaSO_4$					Plasma* adsorbed with $Al(OH)_3$				
Incubation (minutes)	C 1:25	P 1:25	C 1:50	P 1:50	C 1:25	C 1:25	P 1:25	C 1:50	P 1:50	C 1:25
1	59	60	58	53	61	59	72	66	65	68
3	46	41	54	52	47	52	69	64	63	60
5	33	22	53	44	38	42	61	61	60	45
7	18	12	45	32	17	32	58	59	55	30
8	13	11	41	22	14	26	—	—	46	25
9	11	10	30	18	12	24	38	41	42	22
10	9.6	8.8	26	13	9.4	19	31	34	32	19
11	9.4	8.0	21	11	9.8	16	24	29	30	17
12	9.6	8.6	17	10.4	9.6	15	17	23	25	15
13	†	†	15	10.6		14	15	19	20	15
14			14	10.8		15	15	17	18	15
15			14	†		†	15	17	17	15
16			†				†	†	†	
† 50 per cent	14	12	18	14		21	20	29	30	
† 33 per cent	19	16	—	19		30	32	—	—	
Approximate yield (per cent)	100	150	100	180		100	100	100	100	
Half-time (minutes)	7½	7	11½	9½		9½	11½	11	11	

* C = control plasma, P = plasma from patient

† At this time samples were diluted for determination of 50 and 33 per cent values.

TABLE 2: *Results in Thromboplastin Generation Test on Plasma Containing Excessive AHG*

Incubation (minutes)	Clot Times (seconds)									
	Plasma* adsorbed with $BaSO_4$					Plasma* adsorbed with $Al(OH)_3$				
	C 1·25	P 1·25	C 1.50	P 1.50	C 1:25	C 1.25	P 1:25	C 1:50	P 1:50	C 1·25
1	56	52	52	56	52	62	55	54	53	72
3	50	44	51	54	49	58	52	51	—	70
5	45	28	51	41	43	58	28	51	47	62
6		20								
7	37	12	45	28	35	56	22	49	36	60
8	28	9·6	43	21	29	—	14	—	26	55
9	19	8·2	37	13	21	52	11	47	20	50
10	12	8·2	30	10·4	14	52	11	45	16	48
11	11	8·4	24	9·2	12	43	10	43	14	45
12	10	†	20	9·2	11	42	10	—	11·6	40
13	10		16	†	10·4	39	†	41	11·8	37
14	†		15	9·6	10·4	35		—	11·8	37
15	11		15			30		35	†	32
16						27		33		25
17						22		29		21
18						20		25		21
19						21		25		
20						†		†		
† 50 per cent	15	11	19	12		25	14	34	18	
† 33 per cent	19	15	24	17		32	20	59	24	
Approximate yield (per cent)	*100*	*200*	*100*	*225*		*100*	*250*	*100*	*300*	
Half-time (minutes)	9½	7½	12	9		16½	8	15½	9½	

* C = control plasma, P = plasma from patient.

† At this time samples were diluted for determination of 50 and 33 per cent values

of incubation; at 1:50 dilution, 13 to 16 seconds after 11 to 16 minutes The clot times of normal plasma adsorbed with aluminum hydroxide are significantly longer than those of plasma adsorbed with barium sulfate (see tables).

Precautions and Sources of Error: (1) Deterioration of reagents during the test may be minimized by keeping all reagents in a metal test tube rack in a pan filled with ice cubes except when the reagents are actually in use Plasma and serum dilutions are made just before use.

(2) Altered pH, particularly acidity, seriously lengthens the clot times; use of buffered saline tends to prevent this.

(3) If the Dowex resin is not washed thoroughly with saline (incomplete conversion from the hydrogen phase to the sodium phase), it is acidic and hemolyzes the erythrocytes, the prothrombin time then tends to be lengthened. "Dowex plasma" should be discarded if its prothrombin time is more than 3 or 4 seconds longer than that of normal oxalated plasma

(4) Commercial Inosithin appears to be stable indefinitely in its granular form at room temperature; the 5 per cent suspension may deteriorate unless frozen, and once it is diluted to 0 025 per cent, unused remainders are discarded It is critical that the initial suspension of Inosithin in buffered saline be as homogeneous as possible.

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6. Determination of the Partial Thromboplastin Time (PTT)*

R. D. LANGDELL, R. H. WAGNER and K. M. BRINKHOUS

Object of the Test: This test is useful in detecting a deficiency of any of several plasma procoagulants as well as in recognition of circulating inhibitors. The method has been used most extensively as a screening procedure for the detection of "bleeders," but has also been used as a measure of the effectiveness of plasma therapy in hemophilia and hemophilioid diseases and as the basis for quantitative assay procedures for several of the plasma procoagulant factors, including antihemophilic factor (AHF, factor VIII) and PTC (factor IX).

Principle. Certain thromboplastins such as cephalin are unable to compensate completely for the plasma clotting defects of hemophilia and most of the hemophilioid states. These thromboplastins have been termed "partial thromboplastins" The one-stage plasma clotting test performed with the use of a partial thromboplastin is called the *partial thromboplastin time* (PTT)

Apparatus. (a) Syringes and needles. (b) Graduated centrifuge tubes. (c) Glass tubes 10 x 75 mm. are used in determining the clotting times. Tubes of larger size are necessary for storage of reagents. (d) A supply of serologic pipettes, 0.2 and 1.0 ml. (e) Centrifuge. (f) 37°C water bath. (g) Stop watch, preferably operated with a foot pedal. (h) Refrigerator (i) Freezer Any standard type freezer cabinet (−20°C or lower) is used for storage of thermolabile reagents.

Reagents Best results are obtained when the reagents itemized below are used:

(a) 0 11 M (3 2 per cent) sodium citrate

(b) 0 10 M (1 34 per cent) sodium oxalate.

(c) 0 02 M calcium chloride

(d) Test and control plasmas It is essential for interpretation of results that blood from an established normal individual be obtained at the same time and under the same conditions as is the test sample. Obtain blood by venepuncture and mix immediately in a graduated centrifuge tube with 0 11 M sodium citrate in a ratio of one part citrate solution to nine parts whole blood Sodium oxalate solution (0 10 M) may be substituted

* Investigations upon which this method is based were supported in part by research grants H-1648 and H-06350, and by fellowship grants, GM-K3-1147 and GM-K3-15,091

for the citrate. Plasma is obtained by centrifugation. Citrated plasma may be stored at −20°C. for several days before testing.

(e) Partial thromboplastin. Any of a number of preparations may be used, including partial thromboplastins made available commercially. Platelet suspensions, vegetable phosphatides, and crude cephalin all act as partial thromboplastins. Cephalin from bovine, canine or human brain tissue, properly prepared, is a reliable partial thromboplastin. The directions for preparation of this material are as follows:

(1) Wash the fresh brain under tap water. Remove the meninges, accessible blood vessels, brain stem, and cerebellum. Brain tissue can be kept in the frozen state at −20°C. for several weeks prior to being processed

(2) Cut about 100 Gm. of brain tissue into small pieces. Place in a mortar and cover the tissue with acetone. Gently mash the brain tissue under acetone with a pestle. Decant the acetone when it becomes cloudy. Add more acetone and repeat the procedure until the acetone remains clear The dehydrated tissue, which is flaky, may be used immediately in the next step of the procedure or it may be stored at −20°C for several weeks. (It may also be used to prepare thromboplastin suspensions for the prothrombin time test.)

(3) Ether extraction. The acetone-dehydrated tissue is extracted with ether as follows Grind the tissue with clean sand, and place the mixture in an Erlenmeyer flask. Add sufficient ether to cover the material. After mixing, stopper the flask and let stand overnight at room temperature. It is important to add sufficient ether so that the material remains covered throughout this period. Filter and discard the solid material If the filtrate is turbid, repeat the filtration step. Evaporate the ether filtrate to dryness under vacuum, using a rotating evaporator. Wash the residue twice with 40 ml acetone and twice with 40 ml. boiling acetone Air-dry the residue. The yield is usually 2 to 3 Gm of a waxy white material

(4) Preparation of suspension To 3 Gm of the product add 3 ml. of normal saline (0 154 M NaCl) Emulsify with the aid of a sturdy stirring rod Bring the volume to 100 ml by slow addition of normal saline with mixing This 3 per cent suspension is stored at −20°C. in 1 ml lots. Make up a 0 3 per cent suspension with normal saline. This can be stored at −20°C. until used in the test

Steps in the Procedure: (a) Place in a 37°C water bath sufficient partial thromboplastin suspension (0 3 per cent cephalin or its equivalent) and 0 02 M $CaCl_2$ to perform the desired number of tests At least 0.4 ml. of each reagent is necessary for each plasma to be tested.

(b) Plasmas to be tested are kept at room temperature (28°C. ± 1°).

(c) To a clean dry glass tube (10 x 75 mm.) add 0.1 ml. plasma and 0 1 ml. partial thromboplastin suspension Mix and place in 37°C. water bath for 30 seconds. Add 0 1 ml 0 02 M $CaCl_2$ and mix Start timer immediately on addition of $CaCl_2$. The tube is gently agitated until the first visible sign of a clot appears; the clotting time is recorded in seconds. Each test is done in triplicate

Expression of Results· The triplicate values are averaged The mean clotting time in seconds is given for the control and for each of the test plasmas.

Range of Values· The values obtained with normal human plasma depend on the partial thromboplastin preparation used With cephalin, values in the range of 65 to 80 seconds are usually obtained for normal plasma. In a series of 206 normal plasmas tested with "crude cephalin," the mean clotting time was 76 seconds with a standard deviation of 13 seconds

The following empiric rules are used in interpreting the prolonged PTT of test plasmas (a) When the partial thromboplastin time of the test plasma is within 10 seconds of the control time, it should be considered as normal (b) When the partial thromboplastin time of the test plasma is within 11-20 seconds of the control time, it should be considered as probably abnormal (c) If the partial thromboplastin time of the test plasma is 20 or more seconds longer than the control, it is definitely abnormal and a significant deficiency of one plasma procoagulant is present

Additional Procedure if the PTT Test is Prolonged A series of additional PTT tests may be performed to establish a presumptive diagnosis An abnormally long PTT in the presence of adequate fibrinogen indicates either a deficiency in one or more plasma procoagulants or an excess of circulating inhibitors The following additional determinations are made

(a) Inhibitor test· Mix equal parts of test plasma and normal control plasma Use 0 1 ml. of this mixture as the plasma in the PTT test A long PTT is indicative of excess circulating inhibitor

(b) Combined PTT test and prothrombin time test (PT) for procoagulant deficiency (1) A prothrombin time is done on the test plasma (2) A *Modified PTT* test is done using 0 1 ml of a mixture of equal parts of test plasma and $BaSO_4$-treated normal plasma (oxalated plasma adsorbed with 100 mg. $BaSO_4$ per ml plasma) (3) The results are interpreted as follows.

PT	Modified PTT	Probable Deficiency
normal	normal	AHF (Factor VIII) PTA (factor XI) or Hageman (factor XII) deficiency should also be considered
normal	prolonged	PTC (Factor IX)

PT	Modified PTT	Probable Deficiency
prolonged	prolonged	Stuart factor (Factor X) A two-stage prothrombin determination should be done to rule out prothrombin deficiency
prolonged	normal	Factor V

Note: Deficiency in factor VII does not prolong PTT test.

(c) Specific procoagulant deficiency test: On the basis of the results above, a presumptive diagnosis is reached. A confirming PTT test is done, using a mixture of test plasma and a plasma known to be deficient in a single procoagulant, e.g., Stuart plasma. A panel of lyophilized deficient plasmas is maintained for this purpose If the PTT under these conditions remains prolonged, the presumptive deficiency is considered confirmed

Alternate Procedure. Several modifications of the PTT test have been described, including a micromethod Most of the modifications are designed to control the effect of "accelerators" in plasma which shorten the PTT. Accelerator formation has been decreased by use of silicone-coated glassware and low temperatures (4°C). More commonly used are modifications designed to increase formation of accelerators by contact with silica minerals such as kaolin, or by addition of preformed "accelerators," such as activation product, serum $BaSO_4$ eluate or dilute thrombin. One modification is described:

(a) Partial thromboplastin time with silica activated plasma. The plasmas, both test and control, are activated with a silica suspension (3 Gm per cent kaolin, J T Baker, or 5 Gm per cent Celite, Johns Manville Co, or 3 Gm. per cent White Rock Silica in 0.05 M Tris buffer, pH 7 05). The test is performed by adding to 0 1 ml plasma an equal volume of silica suspension and incubating for 5 minutes at 37°C before adding the partial thromboplastin The remainder of the test is performed without change The total volume of the final clotting mixture is 0 4 ml Values for normal plasma are usually in the range of 39 to 46 seconds

Precautions and Sources of Error (a) The test is deceptively simple. Reproducible results can be obtained only by adhering to the principles of good laboratory technic Reagents should be placed in the 37°C water bath only a few minutes before the test is performed It is essential that the plasmas be carefully prepared, and that the control plasma be treated exactly the same as the test plasmas

(b) Accurate use of the pipette is of major importance Standard measurement of 0.1 ml volumes is obtained by using the final 0 1 ml of a 0 2 ml serologic pipette, graduated to the tip Solutions should be drawn into the pipette and allowed to drain at least 3 times prior to making the final measurement; this assures adequate mixing of the reagent and unifor

coating of the pipette. The reagent is delivered into the tube by "blowing out" the contents, avoiding air bubbles

(c) The results are influenced by the factor(s) activated by contact with glass Plasma tested within a few minutes after collection will not clot as rapidly as the same plasma allowed to be in contact with glass for 45 minutes. Likewise, plasmas collected with silicone-coated syringes and stored in silicone-coated tubes will give a long PTT. Determinations done on plasma allowed to be in contact with glass at least 45 minutes from the time of venepuncture will be consistent. The procedure is very sensitive to variations in factor V; oxalated samples, particularly if kept too long prior to testing, will give an abnormally long PTT.

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7. Estimation of Antihemophilic Activity by the Partial Thromboplastin Time Technic*

R. D. LANGDELL, R. H. WAGNER and K. M. BRINKHOUS

Object of the Test To determine the antihemophilic activity of plasma or plasma fractions The results are expressed in relation to the antihemophilic activity of a normal plasma of known antihemophilic factor (AHF, factor VIII) concentration. This procedure is useful because it is relatively simple to perform, yet gives a good estimate of antihemophilic activity if certain precautions are taken The test has been of value in both diagnostic and plasma fractionation studies [1, 2]

* Investigations upon which this method is based were supported in part by research grants H-1648 and H-06350, and by fellowship grants GM-K3-1147 and GM-K3-15091

PT	Modified PTT	Probable Deficiency
prolonged	prolonged	Stuart factor (Factor X) A two-stage prothrombin determination should be done to rule out prothrombin deficiency
prolonged	normal	Factor V

Note: Deficiency in factor VII does not prolong PTT test

(c) Specific procoagulant deficiency test: On the basis of the results above, a presumptive diagnosis is reached A confirming PTT test is done, using a mixture of test plasma and a plasma known to be deficient in a single procoagulant, e.g., Stuart plasma. A panel of lyophilized deficient plasmas is maintained for this purpose. If the PTT under these conditions remains prolonged, the presumptive deficiency is considered confirmed

Alternate Procedure Several modifications of the PTT test have been described, including a micromethod Most of the modifications are designed to control the effect of "accelerators" in plasma which shorten the PTT. Accelerator formation has been decreased by use of silicone-coated glassware and low temperatures (4°C) More commonly used are modifications designed to increase formation of accelerators by contact with silica minerals such as kaolin, or by addition of preformed "accelerators," such as activation product, serum $BaSO_4$ eluate or dilute thrombin One modification is described.

(a) Partial thromboplastin time with silica activated plasma. The plasmas, both test and control, are activated with a silica suspension (3 Gm. per cent kaolin, J T Baker, or 5 Gm per cent Celite, Johns Manville Co, or 3 Gm per cent White Rock Silica in 0 05 M Tris buffer, pH 7.05) The test is performed by adding to 0 1 ml plasma an equal volume of silica suspension and incubating for 5 minutes at 37°C before adding the partial thromboplastin The remainder of the test is performed without change The total volume of the final clotting mixture is 0 4 ml Values for normal plasma are usually in the range of 39 to 46 seconds

Precautions and Sources of Error (a) The test is deceptively simple Reproducible results can be obtained only by adhering to the principles of good laboratory technic Reagents should be placed in the 37°C water bath only a few minutes before the test is performed It is essential that the plasmas be carefully prepared, and that the control plasma be treated exactly the same as the test plasmas

(b) Accurate use of the pipette is of major importance Standard measurement of 0 1 ml volumes is obtained by using the final 0 1 ml of a 0 2 ml serologic pipette, graduated to the tip Solutions should be drawn into the pipette and allowed to drain at least 3 times prior to making the final measurement; this assures adequate mixing of the reagent and uniform

10. Barium sulfate treated plasma. It is preferable to carry out this procedure at 4°C. Add 100 mg Merck U.S.P. or Baker and Adamson reagent $BaSO_4$ powder per ml. oxalated plasma. Adsorb for 30 minutes, with occasional mixing. Centrifuge at about 3000 g for 30 minutes. Withdraw the supernatant plasma. This plasma should not clot when mixed with thromboplastin and calcium.

11. Partial thromboplastin. Any of a number of partial thromboplastins may be used in the assay. A crude cephalin preparation, as described for the partial thromboplastin time test (page 104), or a suitable commercial partial thromboplastin is ordinarily employed.

Steps in Performance of the Test: Test and control plasmas are treated with barium sulfate before use.

(a) Place hemophilic plasma substrate in the ice bath Bring 0.3 per cent crude cephalin and calcium-imidazole solutions to 28°C. in the water bath

(b) Prepare 0 015 per cent cephalin suspension by diluting the 0.3 per cent suspension 1 to 20 with normal saline

(c) Using oxalated saline as a diluent, prepare 1 per cent, 2.5 per cent, 5 per cent and 10 per cent dilutions of test and control plasmas. One ml. of each dilution is adequate When plasma fractions are being tested, previous dilutions must be considered in preparing these solutions. Variations in plasma dilution caused by varying hematocrits may be corrected for in the final calculations

(d) The diluted plasmas are tested by mixing, in order:

0 1 ml hemophilic plasma substrate
0.1 ml diluted test or control plasma
0 1 ml 0 015 per cent cephalin suspension
0 1 ml calcium-imidazole solution

Determine time elapsing from addition of calcium-imidazole until clot forms

Clotting times are determined for each dilution of both test and control plasmas This should be done in a systematic order rather than at random Consistent results are obtained if determinations are done as follows

1 10 per cent control plasma
2 10 per cent test plasma
3 5 per cent control plasma
4 5 per cent test plasma.
5 2 5 per cent control plasma
6 2 5 per cent test plasma
7 1 per cent control plasma
8 1 per cent test plasma

Principle Underlying the Test: The relatively slow clotting of hemophilic plasma with a partial thromboplastin can be accelerated by the addition of small amounts of normal plasma Within limits, the shortening of the partial thromboplastin time of hemophilic plasma is proportional to the amount of normal plasma present. By comparing the relative effectiveness of a known normal plasma and the test material, the corrective effect (antihemophilic activity) may be expressed as per cent of normal.

Apparatus (1) Syringes and needles (2) Centrifuge tubes (3) Centrifuge. (4) Refrigerator. (5) Balance. (6) Glass tubes, 10 x 75 mm, are used in determining clotting times. Tubes of larger size are necessary for storage of reagents. (7) 28°C water bath. (8) Serologic pipettes A supply of 0.2, 1 and 5 ml. pipettes is needed. (9) Stop watch, preferably one that can be operated with a foot pedal. (10) Icebath It is convenient to use a small Dewar flask filled with ice and water. (11) Freezer. Any standard type freezer cabinet capable of maintaining a temperature of −20°C. or lower is useful The freezer is used for storage of thermolabile reagents

Reagents Best results are obtained when the reagents itemized below are used:

1. 0 11 M sodium citrate
2. 0.10 M sodium oxalate
3. 0 11 M calcium chloride
4. 0 154 M sodium chloride (normal saline)
5. Imidazole buffer, pH 7 2 Weigh out 1 72 Gm C.P. imidazole (Edcan Laboratories, South Norwalk, Conn) Dissolve in approximately 90 ml 0 1 N HCl Adjust to pH 7 2, dilute to 100 ml Store in freezer.
6. Oxalated saline. Mix 1 part 0 10 M sodium oxalate with 5 parts of normal saline
7. Calcium-imidazole solution Mix 7 parts 0 11 M calcium chloride, 6 parts imidazole buffer pH 7 2, and 5 parts normal saline. Store at −20°C in 10 ml lots
8. Hemophilic plasma substrate Obtain blood from a known hemophiliac using the two-syringe method Avoid air bubbles in collection Mix immediately with 0 11 M sodium citrate in a ratio of 1 part sodium citrate to 8 parts whole blood Centrifuge at about 3,000 g for 20-30 minutes Withdraw the supernatant plasma after determining the hematocrit Keep at 4°C until just before use If the plasma is not to be tested within 1-2 hours after venepuncture, store immediately in freezer.
9. Oxalated plasma Obtain blood by venepuncture, mix immediately with 0.10 M sodium oxalate in a ratio of 1 part sodium oxalate to 9 parts whole blood The rest of the procedure is the same as in (8) above

of the antihemophilic activity of the test sample in terms of per cent of the control.

Equivalent Concentration of Test Plasma (per cent)	AHF Activity as Per Cent of Control
1.4	71
3 5	71
6 9	72
Average:	71

Alternative Procedures: (a) Shorter clotting times and sharper end points are obtained by the addition of a very dilute solution of "accelerators" to the 0 015 per cent cephalin suspension The accelerator amounts necessary vary somewhat with the reactivity of the standard reagents. To 5.9 ml. of the cephalin suspension is added 0.1 ml of: topical thrombin, 0 5 unit per ml ; or Russell viper venom, 0 04 μg. per ml.; or serum accelerator, appropriately diluted. (The modified procedures allow more rapid testing, but should not be attempted until one is familiar with the assay.)

(b) When the test sample contains a higher concentration of antihemophilic activity than the control, it is more convenient to determine the concentrations of test plasma that would give the same clotting times as 2.5, 5, and 10 per cent control plasma

(c) Canine hemophilic plasma may be used as a substrate instead of human hemophilic plasma However, the PTT of canine hemophilic plasma, unlike human hemophilic plasma, is affected but little by the addition of normal human plasma By increasing the ionic strength of the canine hemophilic plasma, its PTT is further lengthened. The addition of normal human plasma in small increments to the hypertonic stubstrate will give values similar to those plotted in figure 1. The canine hemophilic citrated plasma is diluted with equal volumes of 1.7 per cent NaCl solution before its use in the assay as the hemophilic plasma substrate [8]

Normal Range of Values In a control group of 28 normal adults of both sexes the range was 68 to 165 per cent of the mean, and the standard deviation was ± 28 per cent.

Persons with classical hemophilia have little or no detectable antihemophilic activity

Precautions and Sources of Error (1) Because of the wide range of normal values, careful selection of plasma to be used as a control is required Best results are obtained by using the plasma of an individual whose antihemophilic activity is known in relation to the mean of a large group of normal persons, or by using pooled plasmas from several healthy adults

(e) Repeat(d) twice so that three clotting times are obtained for each dilution of both test and control plasma.

Calculations: The calculations are illustrated by means of an assay of normal human plasma.

(a) Calculate the average clotting time for each plasma dilution

Sample	Av. Clotting Time (sec.)
10 per cent control plasma	130
10 per cent test plasma	141
5 per cent control plasma	153
5 per cent test plasma	165
2 5 per cent control plasma	176
2.5 per cent test plasma	187
1 per cent control plasma	203
1 per cent test plasma	214

(b) Plot clotting time against plasma concentration on semi-log paper. The plasma concentration is represented on the log scale.

(c) Determine by interpolation the concentrations of test plasma that would give the same clotting times as 1, 2.5 and 5 per cent control plasma

Divide control plasma concentration by the equivalent test plasma concentration and multiply by 100 The average of the results is an expression

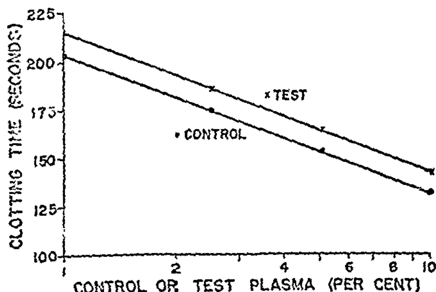


Fig 1 —Graph illustrating method of plotting data for calculation of antihemophilic activity in test sample

of the antihemophilic activity of the test sample in terms of per cent of the control

Equivalent Concentration of Test Plasma (per cent)	AHF Activity as Per Cent of Control
1.4	71
3.5	71
6.9	72
Average:	71

Alternative Procedures: (a) Shorter clotting times and sharper end points are obtained by the addition of a very dilute solution of "accelerators" to the 0.015 per cent cephalin suspension. The accelerator amounts necessary vary somewhat with the reactivity of the standard reagents. To 5.9 ml. of the cephalin suspension is added 0.1 ml of: topical thrombin, 0.5 unit per ml., or Russell viper venom, 0.04 μg per ml.; or serum accelerator, appropriately diluted. (The modified procedures allow more rapid testing, but should not be attempted until one is familiar with the assay.)

(b) When the test sample contains a higher concentration of antihemophilic activity than the control, it is more convenient to determine the concentrations of test plasma that would give the same clotting times as 2.5, 5, and 10 per cent control plasma.

(c) Canine hemophilic plasma may be used as a substrate instead of human hemophilic plasma. However, the PTT of canine hemophilic plasma, unlike human hemophilic plasma, is affected but little by the addition of normal human plasma. By increasing the ionic strength of the canine hemophilic plasma, its PTT is further lengthened. The addition of normal human plasma in small increments to the hypertonic stubstrate will give values similar to those plotted in figure 1. The canine hemophilic citrated plasma is diluted with equal volumes of 1.7 per cent NaCl solution before its use in the assay as the hemophilic plasma substrate.[5]

Normal Range of Values. In a control group of 28 normal adults of both sexes the range was 68 to 165 per cent of the mean, and the standard deviation was $\pm$ 28 per cent.

Persons with classical hemophilia have little or no detectable antihemophilic activity.

Precautions and Sources of Error. (1) Because of the wide range of normal values, careful selection of plasma to be used as a control is required. Best results are obtained by using the plasma of an individual whose antihemophilic activity is known in relation to the mean of a large group of normal persons, or by using pooled plasmas from several healthy adults.

(2) Uncontrolled amounts of thrombin or serum accelerator(s) shorten the partial thromboplastin time of hemophilic plasma without regard to the amount of antihemophilic activity. It is, therefore, essential that the $BaSO_4$ treatment remove these factors quantitatively from the materials to be tested.

(3) As the reactivity of the hemophilic plasma substrate may vary, a control must be run with each test sample.

(4) The antihemophilic activity of plasma may decrease on standing. Therefore, after the plasma dilutions are made, determinations must be performed promptly.

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8. Estimation of Antihemophilic Activity by the Prothrombin Utilization Technic

J. B. GRAHAM, G. D. PENICK and K. M. BRINKHOUS

Object of the Test To determine the antihemophilic activity of plasma or plasma fractions The results are expressed in relation to the antihemophilic activity of a normal plasma of known AHF (antihemophilic factor) concentration

Principle In classic hemophilia the prothrombin of shed blood is converted to thrombin very slowly [1] After a normal blood transfusion[1] or after addition in vitro of normal plasma to the hemophilic blood, the rate at which prothrombin is converted to thrombin is greatly accelerated This corrective action is attributed to the presence in normal blood plasma of a protein, antihemophilic factor (AHF) Within limits, the amount of prothrombin consumed in the whole hemophilic blood is linearly proportional to the amount of AHF added. Thus, the amount of AHF required to convert 50 per cent of the prothrombin in a sample of hemophilic blood is the same, regardless of the source of the AHF By comparing the amount

of test material (AHF) required to cause 50 per cent conversion of prothrombin in the hemophilic blood with the amount of control plasma required to produce the same change, the amount of AHF in the unknown can be expressed in per cent of the control AHF of plasma is not adsorbed by $BaSO_4$,[2] whereas serum accelerator factors which may influence the prothrombin conversion rate in the presence of AHF are adsorbed For this reason, materials tested for AHF are subjected to preliminary $BaSO_4$ adsorption.

Reagents and Apparatus Required: (a) Reagents and equipment, as outlined for the two-stage determination of prothrombin (page 159) and the prothrombin utilization test (page 165).

(b) An available supply of blood from classic hemophilia, either human or canine

(c) Plasmas from control and test subjects. Mix 9 parts of blood with 1 part of 0 10 M sodium oxalate solution Centrifuge at 3000-5000 g for 10-15 minutes and obtain the oxalated plasma. Mix with powdered $BaSO_4$ in the ratio of 100 mg for each ml plasma Stir periodically At the end of 30 minutes centrifuge at about 3000-5000 g for 15 minutes. Dialyze the adsorbed plasma for 2 hours with continuous agitation in cellulose dialyzer tubing (Arthur H Thomas, Philadelphia) against four changes of citrated saline (1 part 0 11 M sodium citrate solution plus 99 parts physiologic saline, i e, 0 154 M NaCl) Centrifugation, adsorption and dialysis are carried out at 4°C

(d) 10 x 75 mm test tubes, graduated at the 1 25 ml mark.

Steps in the Assay (a) Collect plasmas as described above Record hematocrit values for each blood sample Also record volume of each plasma before and after dialysis

(b) Place 0 15 ml imidazole buffer, pH 7 2, into each of four series of eight graduated test tubes

(c) Prepare serial two-fold dilutions of the test and control samples, using saline as diluent For human plasma, the series consists of dilutions of 1 2, 1.4, to 1 64 Add 0 1 ml. of each dilution of each sample to successive and respective members of the series prepared in (b) This is done in duplicate for each dilution Each tube now contains a 0 25 ml volume of reagents The remaining two tubes in each series are used as controls, 0 1 ml saline is placed into each

(d) Collect hemophilic blood by venepuncture, without anticoagulant, in a silicone-treated syringe Immediately place 1 ml whole hemophilic blood in each tube containing the buffer and diluted plasma Sufficient blood should be obtained to fill each tube and to provide a sample from which the hematocrit value can be obtained 0 15 ml 0 11 M sodium citrate

is immediately added to the first pair of control tubes, the contents mixed and centrifuged

(e) Invert the other tubes once for mixing and place in a 28°C. water bath. At the end of 30 minutes, add 0 15 ml. 0.11 M sodium citrate solution to each tube. Gently loosen clot with an applicator stick. Centrifuge at once at 3000-5000 g for 10 minutes.

(f) Remove sera by aspirating with a capillary pipette and attached rubber aspirating bulb. Pool the supernatant serum from each pair of duplicate tubes.

(g) Determine the prothrombin unitage of each pooled sample by the two-stage method The incubated pair of saline controls is used to verify the conversion defect in the hemophilic blood These should have a residual prothrombin value of 100 ± 10 per cent. If the assay is not done immediately freeze (−20°C) samples.

Calculations: *(a) The object of the calculations is to express the amount* of AHF in the test plasma in terms of the control First, obtain the volume of undiluted test and control samples which, when added to 1.0 ml. volume of whole hemophilic blood, gave a prothrombin half-life of 30 minutes (50 per cent prothrombin converted in 30′). Using the hematocrit value of the whole hemophilic blood, the volume is then expressed as ml. per ml. native hemophilic plasma AHF concentration in the test sample is expressed in terms of per cent of activity of the control. This is calculated as follows:

$$\frac{\text{ml control plasma per ml native hemophilic plasma}}{\text{ml. test sample per ml native hemophilic plasma}} \times 100$$

$$= \text{per cent AHF in test sample}$$

(b) An example will illustrate the calculations. Test plasma was obtained by drawing 9 ml whole blood and mixing with 1 ml of 0.10 M sodium oxalate solution The hematocrit was 50 per cent The concentration of the plasma was reduced by dilution with oxalate as follows

$$\frac{\text{volume of native plasma}}{\text{volume of oxalated plasma}} = \frac{4.5}{5.5}$$

$$= 0.82 \text{ ml native plasma per ml oxalated plasma}$$

During dialysis, the volume of the adsorbed plasma increased from 5 5 ml. to 5 8 ml Thus, the concentration was reduced further by the factor, $\frac{5.5}{5.8} = 0.95$

The hematocrit value of the hemophilic blood was 45 per cent Thus 0 1 ml. of each dilution was added to 0 55 ml of native hemophilic plasma.

The actual volume of each dilution of the AHF-containing plasma added to 1 ml native hemophilic plasma is calculated as follows.

Oxalate dilution factor (0 82) x dialysis dilution factor (0.95) x volume of diluted sample added to 1 ml hemophilic blood (0.1 ml.) x factor to adjust 1 ml. whole hemophilic blood to 1 ml. native hemophilic plasma (1/0.55) = 0.142 ml. This figure does not take the series of plasma dilutions into account. To obtain the actual volume then, multiply 0.142 by 1/dilution factor. These values are given in the second column in the tabulation below. The residual prothrombin in the hemophilic serum mixed with that dilution is indicated in the third column.

Plasma Dilution	ml Plasma/ml Native Hemophilic Plasma	Residual Prothrombin Per Cent
1:2	0 071	<10
1·4	0 036	<10
1·8	0 018	35
1·16	0 009	58
1 32	0 0045	68
1·64	0 0023	76
Control	0 0023	100

By plotting the figures in columns 2 and 3 on rectangular graph paper[3] and interpolating, the ml of the test plasma sample which will give a 50 per cent residual prothrombin level (i e., a prothrombin half-life of 30 minutes) is determined This value is 0 0115 ml

From similar calculations with control plasma, it was found that 0.0092 ml. per ml of native hemophilic plasma gave a prothrombin half-life of 30 minutes The AHF in the test plasma was thus.

$$\frac{0\ 0092 \text{ ml}}{0\ 0115 \text{ ml}} \times 100 = 80 \text{ per cent AHF}$$

Range of Normal Values For man, 50-200 per cent of the mean.

Precautions and Sources of Error (a) Care should be taken to avoid air bubbles and rough manipulations during the collection of the blood, inversion of the tubes, and loosening of the clots

(b) Prothrombin and other adsorbable procoagulants should be removed completely in the $BaSO_4$ adsorption step A rapid check can be made by doing a one-stage prothrombin time determination If clotting occurs in less than 10 minutes, readsorb

(c) An occasional hemophilic patient will be refractory to the corrective action of AHF. It is believed such patients have developed excessive inhibitors Their blood is not suitable for this assay procedure Similarly, blood from a hemophilic subject who has received a transfusion within the

preceding 2 weeks, or blood from a mild hemophiliac with a normal prothrombin conversion rate should not be employed

(d) In work with human plasma, the subject or subjects used as controls should be carefully selected. We test a group of 15 to 20 normal subjects and select one or more individuals with values approximating the mean as our standard reference.

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9. Preparation of Plasma Antihemophilic Factor*

R. H. WAGNER and D. PATE

A simple laboratory procedure is described for the preparation of canine plasma antihemophilic factor (AHF, factor VIII) which is largely free of fibrinogen and the easily adsorbed clotting factors. With the use of this simple method, material has been obtained that has proved useful for both in vivo[1] and in vitro laboratory experiments that required AHF concentrates The method described is based upon careful adsorption of oxalate plasma with $BaSO_4$ or of citrate plasma with $Al(OH)_3$, removal of fibrinogen by treatment with fuller's earth, and precipitation of AHF with dilute alcohol under controlled conditions

Apparatus. (1) Syringes and needles (2) Centrifuge tubes (3) Cold room maintained at a temperature of about 4°C (4) pH meter (5) Lyophilizing equipment (6) Freezer Any standard type freezer cabinet capable of maintaining a temperature of −20°C or lower is satisfactory (7) Special centrifuge such as Adams Sero-Fuge (8) Efficient stirring motors (9) Cold bath maintained at about −2°C Mechanical refrigeration can

* Investigations in this laboratory were supported in part by research grant H-1648 and by fellowship grant GM-K3-1147

be used An eutectic mixture of crushed ice and pre-cooled anhydrous sodium carbonate can be used to maintain a temperature of −2°C. (10) Constant temperature water bath at 28°C. (11) A refrigerated centrifuge or a centrifuge in a cold room.

Reagents: All materials used are of reagent grade unless otherwise specified.

1. 0 10 M sodium oxalate.
2. 0 154 M sodium chloride (normal saline).
3. 0 11 M sodium citrate.
4. Citrated saline, 1 volume 0 11 M sodium citrate plus 5 volumes of 0.154 M NaCl.
5. Barium sulfate, Merck U.S P.
6. Aluminum hydroxide suspension is prepared and washed with distilled water until pH of the supernatant is less than 6.7 Each batch is tested with the prothrombin time test for its efficacy in removal of procoagulants from plasma. The suspension is stored at 4°C
7. Fuller's earth (Florigel) from Floriden Co , Tallahassee, Florida.
8. 0 1 N acetic acid.
9. Topical thrombin (Parke, Davis & Co , Detroit, Mich.) is diluted with 0 154 M sodium chloride to a cencentration of 100 Iowa units per ml. and stored in 0 2 ml lots at −20°C until needed.
10. Special solvent Eight hundred ml of 0 154 M sodium chloride are mixed with 200 ml of 0 11 M sodium citrate and 100 ml of phosphate buffer, pH 7.2, ionic strength 0 055 Final ionic strength of the solvent is 0 237
11. Buffered ethanol (20 per cent) One hundred ml of 95 per cent ethanol, 237 5 ml of solvent described in (10), and 137 5 ml of distilled water are mixed Final ionic strength is about 0 118
12. Plasma (a) Plasma prepared with citrate Obtain blood from a large healthy donor dog by jugular venepuncture, using the two syringe method Use pre-cooled 50 ml syringes containing 5 5 ml of 0 11 M sodium citrate, draw blood to the 50 ml mark, mix immediately and deliver gently into a cold 120 ml plastic centrifuge tube The blood is centrifuged in the cold and the plasma is carefully aspirated and delivered into a cold measuring cylinder

    (b) Plasma prepared with oxalate Blood is obtained in a manner similar to the method described above Five ml of cold 0 10 M sodium oxalate is used in each syringe and blood is drawn to the 50 ml mark
13. Adsorbed plasma (a) $BaSO_4$-treated plasma Plasma prepared with the use of oxalate is adsorbed in the cold room for 30 minutes with 50 mg $BaSO_4$ per ml Mix carefully and frequently to prevent the $BaSO_4$

from settling. Avoid bubbles. Centrifuge for 10 minutes, decant, and repeat the adsorption step with 100 mg. $BaSO_4$ per ml.

(b) $Al(OH)_3$-treated plasma. The proper amount of cold $Al(OH)_3$ suspension (usually 5 ml. per 100 ml. of plasma) is added with stirring to plasma anticoagulated with citrate. The adsorption is carried out in the cold with frequent mixing, and the $Al(OH)_3$ removed by centrifugation for 30 minutes

Steps in the Procedure.

1. *Adsorption of fibrinogen* After adsorption with $Al(OH)_3$ or $BaSO_4$, the pH of the plasma is adjusted slowly with constant stirring to 6 9 with 0 1 N acetic acid. Samples for AHF standards are removed and quick frozen. The plasma is then placed in a beaker in a constant temeprature water bath at 28°C Florigel (30 mg. per ml.) is added to the plasma, mechanical stirring is used to maintain uniform dispersion of the adsorbent The rate of adsorption of fibrinogen must be followed as it varies widely with different plasmas. Every 5 minutes, 1.0 ml. of the plasma Florigel suspension is pipetted into a 10 x 75 mm. test tube and the adsorbent is sedimented within a 45 second period in an Adams Sero-Fuge. Two-tenths ml of the supernatant plasma is pipetted into 0.2 ml. of the stock thrombin solution and the time required for a fibrin clot to form is recorded When the thrombin clotting time reaches the desired time (usually 30 to 40 seconds) for adequate adsorption of fibrinogen, the plasma is removed to an ice bath and the Florigel allowed to settle for several minutes. The plasma is then decanted into centrifuge tubes, leaving behind in the beaker as much Florigel as possible, and the adsorbent is removed by centrifugation

2 *Removal of traces of Florigel* Last traces of Florigel are difficult to remove by centrifugation If they are not removed they contaminate the AHF precipitate. Therefore, the following step is used after the primary removal of Florigel by centrifugation Several ml $Al(OH)_3$ suspension are mixed with the plasma and immediately removed by a second centrifugation This effects almost quantitative removal of Florigel

3 *Alcohol precipitation* The pH of the adsorbed plasma is adjusted to 7 0-7 1 with 0 1 N acetic acid, the volume is measured, and the plasma is cooled to 0°C The 20 per cent alcohol buffer solution is cooled to −2°C The plasma is transferred to a −2°C bath and ½ volume of chilled buffered ethanol is added dropwise with efficient stirring The final ethanol concentration is 6 7 per cent The mixture is allowed to stand for 2 hours in the −2°C bath The precipitate is collected by centrifugation and the supernatant is poured off for other fractionation purposes The

precipitate is washed 3 times with one-fourth the plasma volume of phosphate buffer, pH 7.2, and 0.1 ionic strength, containing successively 7 per cent, 2.5 per cent, and 0 per cent ethanol. The thrice-washed precipitate is dissolved in solvent at 25°C. The solution may need to be clarified by centrifugation. The solution can be shell-frozen and lyophilized. The dry powder is best kept in a vacuum desiccator at −20°C. Dry powder kept at room temperature for several months loses activity. The yield of AHF varies from 25 to 70 per cent and the number of times purified ranges from 50 to 150 times.

4. *Total protein determination.* Protein concentrations of AHF fractions are conveniently determined by measuring the ultraviolet absorption of the solutions in the Beckman DU spectrophotometer at 280 and 320 mμ. The small background reading at 320 mμ is subtracted from the 280 mμ reading, and the protein concentration is calculated using $E^{1\%}_{1cm,\ 280\ m\mu} = 15$. The extinction coefficient of 15 is based on the assumption that AHF fractions contain mainly globulins and that these globulins are typical with regard to their content of aromatic amino acids. Use of this coefficient gives the same protein values as total nitrogen times 6.25.

5. *Assay.* A sample of the dry fraction is dissolved, diluted appropriately and assayed for AHF activity by the one-stage AHF assay (see page 107 in this book). The activity is ordinarily expressed as per cent of the starting plasma. A dry preparation stored in a desiccator in a freezer may also serve as a suitable standard once its activity has been determined in terms of control plasma.

Precautions and Sources of Error.

1. Very careful collection of the blood is essential.

2. Frothing during Florigel, $BaSO_4$, or $Al(OH)_3$ adsorption may be minimized by the addition of a few tiny drops of 2-octanol.

3. Treatment with Florigel for too long a period of time will result in loss of AHF activity. Adsorption with excess $Al(OH)_3$ may cause loss of AHF activity.

4. It is necessary to keep total elapsed time for the preparation to a minimum.

5. Frozen plasma is not a satisfactory starting material, even if it has been given preliminary treatment with $BaSO_4$ in the case of plasma containing oxalate or $Al(OH)_3$ with citrate plasma.

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10. Estimation of PTC (Factor IX) Activity by the Partial Thromboplastin Time Technic*

E. M. BARROW and J. B. GRAHAM

Object of the Test To determine the relative amount of PTC in plasma from normal individuals and from patients suspected of having reduced amounts of this factor

Principle The PTC activity can be detected by the ability of a test plasma to shorten the prolonged partial thromboplastin time (PTT) of a known PTC-deficient plasma In this assay system,[1] all clotting factors are present in adequate amounts with the exception of PTC. By comparing the effectiveness of a normal plasma and the test plasma, the corrective effect (PTC activity) can be expressed as per cent of normal

Apparatus (a) Serologic pipettes 0 2, 1 0 and 5 0 ml. (b) 28°C. water bath (c) 10 x 75 mm test tubes for clotting Larger tubes are necessary for dilution of plasma and storage of reagents (d) Stop watches

Reagents (a) Citrated plasma from a normal individual, or a pool from several persons, is used as control The two-syringe technic is employed in collecting both test and control plasmas Blood is carefully withdrawn from the antecubital vein, without air bubbles, and gently mixed with 3 2 per cent trisodium citrate solution in the ratio of 1 part of citrate to 8 parts of blood The citrated blood is centrifuged for 30 minutes at 3,000 g The plasmas are withdrawn and kept at 4°C until diluted for use in the assay.

(b) Citrated, lyophilized plasma for substrate Citrated plasma is prepared as above from an individual who is severely deficient in PTC, but has normal amounts of other clotting factors Two to 3 ml amounts of

* Investigations upon which this method is based were supported in part by research grant H-06350-01

the plasma are lyophilized, the ampoules being sealed under vacuum and stored at −20°C.

(c) $BaSO_4$-treated normal dog plasma: Blood is obtained by venipuncture and mixed with 0 10 M sodium oxalate in the ratio of 1 part oxalate to 9 parts blood. After centrifugation, the plasma is removed and cooled to 4°C To each ml. oxalated plasma are added 100 mg $BaSO_4$ (Merck) The plasma is adsorbed for 30 minutes with occasional mixing The $BaSO_4$ is removed by centrifugation for 30 minutes at 3,000 g. The adsorbed plasma should not clot when calcium chloride and thromboplastin are added. If a clot does form, repeat the adsorption as above.

This adsorbed plasma is added to the lyophilized, reconstituted substrate plasma to insure the presence of adequate amounts of factors V, VIII and XII which may have been reduced in the substrate plasma by lyophilization and storage.

(d) Cephalin suspension. This is the ether-soluble fraction of human brain prepared according to Rodman et al [2] (see page 103 under Partial Thromboplastin Time) A 3 per cent stock suspension is stored at −20°C.

(e) Imidazole buffer, pH 7 2· 1.72 Gm C.P. Imidazole (Edcan Laboratories, South Norwalk, Conn.) are dissolved in 90 ml 0 1 N HCl. The pH is adjusted to 7 2 and the buffer diluted to 100 ml with distilled water.

(f) Plasma diluent· This is a 1·6 buffered citrated saline prepared by mixing 100 ml 0 9 per cent sodium chloride, 100 ml. imidazole buffer and 40 ml 3.2 *per cent trisodium citrate*

(g) Calcium solution Prepared by mixing 70 ml 1 2 per cent calcium chloride, 60 ml imidazole buffer and 50 ml of 0 9 per cent sodium chloride Store at −20°C

Steps in the Performance of the Test All reagents are kept in the 28°C water bath during testing (a) Reconstitute the lyophilized substrate plasma with imidazole buffer to the original plasma volume Add an equal volume of freshly prepared $BaSO_4$-treated normal dog plasma (b) Dilute the control and test plasmas with buffered citrated saline to the following concentrations 1, 2 5 5, 10 and 20 per cent These dilutions are prepared as follows

% Plasma	Plasma (ml)	Diluent (ml)
20	0 2	0 8
10	*0 2*	*1 8*
5	0 5 (of 10%)	0 5
2 5	0 25 (of 10%)	0 75
1	0 1 (of 10%)	0 9

(c) The cephalin stock suspension is diluted to 0 015 per cent with normal saline. (d) A tube of calcium solution is thawed in the water bath (e)

Each diluted plasma sample is tested by mixing the reagents in the following order:

0.1 ml. PTC substrate
0 1 ml. diluted control or test sample
0 1 ml cephalin
0 1 ml. calcium chloride

A stop watch is started with the addition of the calcium solution The tubes are tilted gently in front of a strong light source and the watch stopped upon the formation of visible fibrin strands. The clotting time for each dilution is recorded. The dilutions are tested in order, i e., all the 20 per cent plasmas, then the 10 per cent plasmas, etc. All samples are tested 3 times and the mean values determined.

Calculations. (a) The mean clotting times for each dilution are plotted against the logarithm of plasma concentration, using 2-cycle semi-logarithmic paper. (b) The best fitting parallel straight lines are fitted to the values for the unknown and the control. (c) The concentrations of test plasma that would give the same clotting times as 1, 2.5, 5 and 10 per cent control plasma are determined by interpolation (d) The control plasma concentration is divided by the equivalent test plasma concentration and multiplied by 100, e g, if the 1 per cent control plasma was equivalent to 1 5 per cent test plasma, the per cent PTC for that concentration would be $\frac{1.0}{1.5}$ x 100 or 66 per cent (e) The average of the results for each plasma concentration is the PTC activity of the test sample in terms of per cent of the control

Normal Range of Values A control population of 58 females[1] showed a range of 50 to 140 per cent PTC Unpublished studies of smaller groups of males indicate a similar range of values.

Precautions and Sources of Error (a) Selection of a control. Since there is such a wide range of PTC activity within a "normal" population, it is wise to select for use as a "normal" control an individual who is found by assay to be approximately 100 per cent of a pooled sample. (b) PTC substrate plasma The individual(s) chosen as donor(s) should have no measurable PTC Also, the plasma should contain, as determined by assay, normal amounts of other clotting factors

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11. Estimation of Factor IX (PTC) Activity of Human Plasma

M. L. KROPATKIN, M. S. HOAG and P. M. AGGELER

Object of the Test: To assay specifically the factor IX activity of human plasma, regardless of the degree of activation produced by previous glass contact.

Principle: The assay is based on the partial thromboplastin time technic of Langdell, Wagner and Brinkhous[1] and the kaolin clotting time method of Proctor and Rapaport.[2] Platelet factor 3 is supplied by a cephalin suspension. Maximum surface contact activation is achieved with celite powder.* Substrate plasma from a patient with severe deficiency of factor IX supplies all other clotting factors except factor IX. Quantitation of an unknown specimen is made by comparing its clotting time in the test with that achieved by dilutions of a standard normal plasma

Reagents and Apparatus Required

(1) Solution of 2 parts 0 1 M citric acid and 3 parts 0 1 M sodium citrate, mixed with all blood specimens in a ratio of 1:9.

(2) Diluting fluids I and II (Hjort et al).[3]

(3) 0 025 M $CaCl_2$ solution

(4) Cephalin-celite reagent equal volumes of. (a) Cephalin suspension, prepared by the method of Milstone as described by Hjort et al.,[3] diluted 1/3, in veronal buffer, pH 7 35 (b) Celite powder,* 1 Gm. suspended in 5 ml 0 9 per cent NaCl solution.

(5) Substrate plasma from a patient with severe congenital deficiency of factor IX, stored in small aliquots at −20°C

(6) Standard normal reference plasma, stored in small aliquots at −20°C

(7) Refrigerated centrifuge.

(8) Clean glass 13 x 100 mm test tubes

(9) 37°C water bath.

(10) Stop watches.

(11) 0 2 ml pipettes graduated in 0 01 ml and 1 0 pipettes graduated in 0 01 ml

(12) Test tube rack in melting ice bath

Steps in Performance of Test. All plasmas are obtained from blood centrifuged at 2000 rpm at 4°C and are stored at −20°C The celite-cephalin reagent is thoroughly resuspended before use The substrate

* Celite powder (Johns-Manville), amorphous diatomaceous SiO_2

Each diluted plasma sample is tested by mixing the reagents in the following order:

0.1 ml. PTC substrate
0.1 ml. diluted control or test sample
0 1 ml. cephalin
0.1 ml. calcium chloride

A stop watch is started with the addition of the calcium solution. The tubes are tilted gently in front of a strong light source and the watch stopped upon the formation of visible fibrin strands. The clotting time for each dilution is recorded The dilutions are tested in order, i e., all the 20 per cent plasmas, then the 10 per cent plasmas, etc. All samples are tested 3 times and the mean values determined.

Calculations· (a) The mean clotting times for each dilution are plotted against the logarithm of plasma concentration, using 2-cycle semi-logarithmic paper. (b) The best fitting parallel straight lines are fitted to the values for the unknown and the control. (c) The concentrations of test plasma that would give the same clotting times as 1, 2.5, 5 and 10 per cent control plasma are determined by interpolation (d) The control plasma concentration is divided by the equivalent test plasma concentration and multiplied by 100, e g , if the 1 per cent control plasma was equivalent to 1 5 per cent test plasma, the per cent PTC for that concentration would be $\frac{1.0}{1.5} \times 100$ or 66 per cent (e) The average of the results for each plasma concentration is the PTC activity of the test sample in terms of per cent of the control.

Normal Range of Values A control population of 58 females[1] showed a range of 50 to 140 per cent PTC Unpublished studies of smaller groups of males indicate a similar range of values.

Precautions and Sources of Error· (a) Selection of a control: Since there is such a wide range of PTC activity within a "normal" population, it is wise to select for use as a "normal" control an individual who is found by assay to be approximately 100 per cent of a pooled sample. (b) PTC substrate plasma. The individual(s) chosen as donor(s) should have no measurable PTC Also, the plasma should contain, as determined by assay, normal amounts of other clotting factors

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Principle: The assay is based on the partial thromboplastin time technic of Langdell, Wagner and Brinkhous[1] and the kaolin clotting time method of Proctor and Rapaport.[2] Platelet factor 3 is supplied by a cephalin suspension. Maximum surface contact activation is achieved with celite powder.* Substrate plasma from a patient with severe deficiency of factor IX supplies all other clotting factors except factor IX. Quantitation of an unknown specimen is made by comparing its clotting time in the test with that achieved by dilutions of a standard normal plasma.

Reagents and Apparatus Required

(1) Solution of 2 parts 0.1 M citric acid and 3 parts 0.1 M sodium citrate, mixed with all blood specimens in a ratio of 1:9.

(2) Diluting fluids I and II (Hjort et al.)[3]

(3) 0.025 M $CaCl_2$ solution

(4) Cephalin-celite reagent: equal volumes of (a) Cephalin suspension, prepared by the method of Milstone as described by Hjort et al.,[3] diluted 1/4, in veronal buffer, pH 7.35 (b) Celite powder,* 1 Gm suspended in 5 ml 0.9 per cent NaCl solution

(5) Substrate plasma from a patient with severe congenital deficiency of factor IX, stored in small aliquots at −20°C

(6) Standard normal reference plasma, stored in small aliquots at −20°C

(7) Refrigerated centrifuge

(8) Clean glass 13 x 100 mm test tubes

(9) 37°C water bath

(10) Stop watches

(11) 0.2 ml. pipettes graduated in 0.01 ml and 1.0 pipettes graduated in 0.01 ml

(12) Test tube rack in melting ice bath

Steps in Performance of Test: All plasmas are obtained from blood centrifuged at 2000 rpm at 4°C and are stored at −20°C. The celite-cephalin reagent is thoroughly resuspended before use. The substrate

* Celite powder (Johns-Manville), amorphous diatomaceous SiO_2

plasma is thawed with constant agitation in the water bath at 37°C. It is then kept in the melting ice bath with all other reagents except the calcium solution, which is placed in the 37°C. waterbath. A standard reference plasma is diluted to concentrations of 10 per cent, 5 per cent, 2 per cent, 1 per cent, 0.5 per cent, 0.4 per cent, 0 3 per cent, 0.2 per cent and 0 1 per cent, with diluting fluids I and II, according to the method of Hjort et al.[8] Unknown plasmas are tested at the 10 per cent and 5 per cent concentrations

One-tenth ml. of resuspended cephalin-celite, 0.1 ml. of substrate plasma and 0.1 ml of diluted test or standard plasma are transferred to a 13 x 100 mm glass tube in the 37°C. water bath and a stop watch started The mixture is gently tilted during the 3-minute incubation period to keep the celite in suspension. At the end of 3 minutes, 0.1 ml of the $CaCl_2$ solution is added and a second stop watch started The tube is tilted until clotting occurs. All tests are done in duplicate and the clotting times averaged

Manner of Expression of Results: The standard reference plasma is tested with each set of unknowns A graph is constructed on log-log paper with clotting time on the ordinate and plasma concentrations on the abscissa. The clotting times of the various dilutions of the standard reference plasma are plotted and two straight lines having different slopes are drawn through the points representing plasma concentrations from 10 per cent to 1 per cent and from 1 per cent to 0 1 per cent. The plasma concentrations are multipled by 10 to convert the factor IX concentration to per cent. The factor IX concentrations of unknown specimens are determined by reference to this graph. The values obtained for the 10 per cent and 5 per cent plasma concentrations of the unknown are averaged When the clotting time of the 10 per cent concentration of the unknown plasma is shorter than that of the standard reference plasma, then only the 5 per cent plasma concentration of the unknown is used in determining its factor IX concentration Concentrations of less than 1 5 per cent may be impossible to determine, since at this point the clotting time of the standard plasma approaches that of the blank A correction factor is applied in plotting the standard reference plasma if its factor IX concentration is not 100 per cent The exact factor IX concentration of the standard reference plasma is determined in a separate experiment which need not be run on the same day unknowns are tested In this determination the standard plasma is tested with a group of at least 10 plasmas obtained from normal subjects, tested individually. The factor IX concentration of the standard plasma is determined from a graph constructed using the mean 5 per cent and 10 per cent plasma clotting times of the group

Normal Range of Values. 50 to 180 per cent (67 to 87 seconds)

Precautions and Sources of Error: (1) The substrate plasma must be obtained from a patient with *severe* congenital factor IX deficiency. The blank clotting time should be at least 160 seconds. Slight traces of factor IX will result in a flat, unusable standardization curve

(2) The substrate plasma cannot be stored in the frozen state for longer than 2 to 3 months and should not be used for more than 1½ hours after thawing, as deterioration of labile clotting factors may occur after these times.

(3) Since celite sediments rapidly, it must be thoroughly resuspended just prior to transferring it from one tube to another.

(4) Unless all glassware is scrupulously clean, poor agreement between duplicate determinations will result.

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12. *Preparation of a Concentrate of Factor IX (PTC) from Serum*

S. S. KAPLAN, P. M. AGGELER and M. S. HOAG

In the proposed method, good concentration of factor IX can be achieved The preparations also contain significant amounts of factor VII, traces of factor X and unknown quantities of factors XI, XII, glass activation product and nonclotting proteins. Traces of unidentified, intermediate clotting products may also be present.

Materials and Reagents

(a) Serum obtained from blood incubated for 24 hours at 37°C. The serum may be stored at −20°C until ready for use

(b) 0 1 M sodium oxalate, mixed with serum in a ratio of 1.9

(c) $BaSO_4$ (Baker), used in a proportion of 10 mg /ml of serum.

(d) K 30 polyvinylpyrollidine (PVP) 30 per cent

(e) 0.9 per cent NaCl solution, for washing
(f) 0.006 M sodium citrate, for washing.
(g) 0.14 M sodium citrate, for elution.
(h) Distilled water.
(i) All centrifugations are performed at 2000 r.p m. at 4°C.

Steps in Procedure. The serum is thawed at 37°C., diluted with sodium oxalate solution and adsorbed with $BaSO_4$, with constant stirring in an ice bath for 45 minutes. After centrifugation for 30 minutes, the supernatant is discarded With constant stirring and at room temperature, the precipitate is washed twice with 0 9 per cent NaCl and once with 0.006 M sodium citrate, in amounts equal to one-third the original volume of the serum used One-sixth original serum volume of 0 14 M citrate solution is added to the washed precipitate and stirred for 30 minutes at room temperature. After centrifugation for 30 minutes, the supernatant is removed and the precipitate eluted again with citrate The precipitate is then discarded and the combined supernates dialyzed for 24 hours against distilled water that is frequently changed. This is followed by dialysis against the PVP solution for 6 to 18 hours, the duration depending upon the volume of eluate and the degree of concentration desired The dialyzed eluate is centrifuged for 10 minutes and used immediately or stored at −20°C.

These concentrates contain approximately 700 mg. protein per 100 ml. and have a factor IX potency 75 times greater than an equal amount of normal plasma Compared to their content of factor IX (considered as 100 per cent), the concentrates contain 3 per cent factor VII and 0.3 per cent factor X They are free of thrombin and factors I, II, V and VIII

Further purification of the concentrate can be achieved by starch block electrophoresis using the method of Kunkle.

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13. Estimation of Factor X (Stuart-Prower Factor) Activity Utilizing the Prothrombin Time Technic*

E. M. BARROW and J. B. GRAHAM

Object of the Test: To determine the relative amount of factor X in plasma from normal individuals and from patients suspected of having reduced amounts of this factor.

Principle: A comparison is made between the ability of an unknown plasma and a control plasma to correct the prolonged prothrombin time (PT) of a plasma specifically deficient in factor X[1]. By comparing the effectiveness of a normal plasma and the test plasma, the corrective effect (factor X activity) can be expressed as per cent of normal

Apparatus: (a) Serologic pipettes: 0.2, 1.0 and 5.0 ml (b) 37°C. water bath. (c) 10 x 75 mm. test tubes for clotting Larger tubes are necessary for dilution of plasma and storage of reagents (d) Stop watches.

Reagents: (a) Citrated control and test plasmas The two-syringe technic is employed in obtaining blood The blood is carefully withdrawn from the antecubital vein, without air bubbles, and gently mixed with 3.2 per cent trisodium citrate solution in the ratio of 1 part of citrate to 8 parts of blood. The citrated blood is centrifuged for 30 minutes at 3,000 g. The plasmas are withdrawn and kept at 4°C. until diluted for use in the assay.

(b) Citrated, lyophilized plasma for substrate Citrated plasma is prepared as above from an individual who is severely deficient in factor X, but who has normal amounts of other clotting factors Two to 3 ml. amounts of the plasma are lyophilized, the ampoules are sealed under vacuum and are stored at −20°C

(c) $BaSO_4$-treated normal dog plasma Blood is obtained by venepuncture and mixed with 0.10 M sodium oxalate in the ratio of 1 part of oxalate to 9 parts of blood After centrifugation, the plasma is removed and cooled to 4°C One hundred mg. $BaSO_4$ (Merck) are added to each ml oxalated plasma The plasma is adsorbed for 30 minutes with occasional mixing. The $BaSO_4$ is removed by centrifugation for 30 minutes at 3000 g The adsorbed plasma should not clot when calcium chloride and thromboplastin are added If a clot does form, repeat the adsorption as above

* Investigations upon which this method is based were supported in part by research grant H-06350-01

This adsorbed plasma is added to the lyophilized, reconstituted substrate plasma to insure the presence of adequate amounts of factor V which may have been reduced in the substrate plasma by lyophilization and storage.

(d) Imidazole buffer, pH 7.2: 1.72 Gm. C.P. Imidazole (Edcan Laboratories, South Norwalk, Conn) are dissolved in 90 ml. 0.1 N HCl. The pH is adjusted to 7.2 and the buffer diluted to 100 ml. with distilled water.

(e) Plasma diluent: This is a 1:6 buffered citrated saline prepared by mixing 100 ml. 0.9 per cent sodium chloride, 100 ml. imidazole buffer and 40 ml 3.2 per cent trisodium citrate.

(f) Thromboplastin: Prepare a saline extract of acetone-dried human brain. This thromboplastin should give a 10-12 second prothrombin time value with normal plasma.

(g) Calcium solution: A 0.02 M calcium chloride solution is used.

Steps in the Performance of the Test: (a) Reconstitute the lyophilized substrate plasma with imidazole buffer to the original plasma volume. Add an equal volume of freshly prepared $BaSO_4$-treated dog plasma (b) Dilute the control plasma with buffered citrated saline as follows: 1:2, designated for this procedure (100 per cent), 1:4 (50 per cent), 1:8 (25 per cent), 1:16 (12 per cent), 1 32 (6 per cent), and 1·64 (3 per cent). (c) Dilute the test plasma(s) with the same diluent to 1:2 and 1:4 concentrations (d) Leave the substrate, control, and test plasmas at room temperature during the performance of the assay (e) Place tubes of thromboplastin and calcium chloride in the 37°C. water bath. (f) Each diluted plasma sample is tested by mixing the reagents in the following order:

0.1 ml factor X-deficient plasma
0 1 ml. diluted control or test plasma
0 1 ml thromboplastin
0 1 ml $CaCl_2$

A stop watch is started with the addition of the $CaCl_2$. The tubes are tilted gently in front of a strong light source and the watch stopped upon the formation of a clot The clotting time of each plasma dilution is recorded (g) Each dilution of control and test plasma is tested twice and the mean clotting time recorded

Calculations Plot the mean clotting times of the control against the plasma concentration on arithmetic graph paper Using the clotting times of the two dilutions of unknown plasma, determine the factor X concentration separately by interpolation from the control curve Average the results

Normal Range of Values Not determined

Precautions and Sources of Error (a) Incomplete adsorption of "prothrombin complex" by $BaSO_4$ in treating the dog plasma will give erroneous

levels. (b) A substrate plasma (factor X-deficient) also having low levels of prothrombin and factor VII will mask the changes in factor X.

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14. Estimation of Factor X (Stuart-Prower) Activity

F. DUCKERT and F. KOLLER

Object of the Test The quantitative determination of factor X activity in plasma, serum and in purified factor X fractions. Factor X, which is indispensable for the intrinsic as well as the extrinsic system, is particularly important for the control of oral anticoagulant therapy. Beside this key position, the factor X quantitative assay method affords a good control of the Quick one-stage assay and similar methods because it is independent of the tissue factor (thrombokinase, thromboplastin). The simultaneous performance of both tests enhances considerably the security of the control of oral anticoagulant therapy

Principle The test is based on the same principle as the Quick one-stage assay method and rendered specific by the addition of an adequate reagent. It is unfortunately impossible at the present time to prepare a factor X-free reagent containing all other clotting factors in suitable concentration The difficulty can be overcome by using a reagent free of factors VII and X and replacing the brain thromboplastin by a Russel's viper venom-cephalin mixture. In the presence of the venom-factor, coagulation is independent of factor VII, which is therefore not required in this particular test system The interference of other clotting factors is eliminated by diluting the plasma and serum to be tested

Reagents

1 Oxalated or citrated plasma One part sodium oxalate 0.1 m, or trisodium citrate 0.1 m, and 9 parts whole blood are rapidly mixed and then centrifuged Serum Whole blood is allowed to clot at room temperature and then incubated for 2 hours at 37°C Since factor X is quite stable, plasma and serum may be stored for as long as 24 hours before testing.

2 Buffer solution. The veronal-acetate stock solution is obtained by dissolving 9 174 Gm. $CH_3COONa{\cdot}3\ H_2O$ and 14 714 Gm Na diethyl-

barbiturate (veronal Na) in 500 ml distilled water. Buffer solution. 250 ml. stock solution, 200 ml. 4.25 per cent NaCl, 217 ml HCl, 0 1 N and 683 ml. distilled water are mixed together. A few drops of toluene are added to prevent contamination. The final pH is 7.35 and the solution is isotonic

3. Filtered bovine plasma. This is the factor VII- and factor X-free reagent. Whole bovine blood from the open jugular vein is collected directly in a 3-liter bottle containing the 0.1 M sodium oxalate solution (1 part oxalate and 9 parts blood). The oxalated bovine plasma is obtained by centrifugation at 1500 g for 30 minutes The plasma (1500 ml) is poured into a 2-liter original Seitz filtration apparatus (Seitz Werke, Bad Kreuznach, Rheinland, Germany) fitted with two asbestos filters (filter pads from Filtrox, St Gall, Switzerland) The pad No. 7 containing 30 per cent asbestos is placed under the coarser pad No 5 containing 20 per cent asbestos. The filtration is carried out at room temperature and the speed maintained at 40 drops per minute by application of air pressure. The pressure has to be increased during filtration. Too high filtration speed results in poor adsorption of factor X, at lower speed the filter pads remove large amounts of prothrombin. The first 200 ml. are discarded. The filtrate is then collected in 50 ml fractions Each fraction is tested for its prothrombin content (specific activity) which should be above 30 per cent of the normal value, and for the blank value (see below) which should be longer than 80-90 seconds The fractions with adequate true prothrombin levels and blank values are mixed together and frozen at −20°C. in small portions suitable for half-a-day work

4 Crude cephalin According to Hjort, Rapaport and Owren,[2] 400 Gm blood and membrane-free human brain are ground with 300 ml. acetone and centrifuged. The supernatant is discarded and the operation repeated 5 times with fresh acetone The sediment is extracted overnight at room temperature with 1 8 liters of ethyl ether The supernatant is separated and the ether evaporated The dry residue is then extracted twice with 900 ml acetone for 15 minutes, and finally dissolved in 200 ml. ether The ether is evaporated under vacuum The final product looks like a thick viscous and brownish cream Nine and three-tenths gm of this cream are suspended in 200 ml buffer solution, and centrifuged at 700 g for 20 minutes After discarding the sediment, the suspension is centrifuged 10 minutes at 100 g The supernatant contains about 2 8 Gm crude cephalin in 100 ml This suspension is kept frozen at 20°C It is stable for years

5 Russel's viper venom (RVV) Stypven, a Burroughs, Wellcome and Co, (London) preparation, is used Each vial contains 0.5 mg. dry substance The original solvent containing phenol must be discarded; otherwise the mixture with cephalin is unstable after freezing.

6. RVV-cephalin mixture. The crude cephalin suspension is diluted 1:60 in buffer. The Stypven-dry substance is dissolved in this cephalin suspension. The final Stypven concentration amounts to 1:200,000 (w/v). The suspension is homogenized in a Potter glass apparatus and then frozen at −20°C. in small portions It is stable for months in the frozen state

7. Calcium chloride A 1.40 M solution is used.

Performance of the test. The test is carried out in a water bath at 37°C The frozen reagents are rapidly thawed The factor VII- and factor X-free reagent is kept at room temperature, the Stypven-cephalin mixture and the calcium chloride are kept at 37°C. Both reagents are stable for 3-4 hours Two parallel assays are performed for each determination.

The following solutions are added successively into two tubes placed in the water bath·

0.1 ml. factor X-free reagent
0.1 ml plasma or serum diluted 1 10 in buffer
0.1 ml. RVV-cephalin mixture

After 30 seconds incubation at 37°C., 0 1 ml $CaCl_2$ 1.40 M, is blown into the mixture and a stop watch started The clotting time is recorded using a bent platinum wire for determining the end point

Determination of the blank The veronal-acetate buffer replaces the plasma or serum dilution in the test. The clotting time should be longer than 80-90 seconds Shorter clotting times indicate that the adsorption on the asbestos filters is incomplete

Calibration Curve The calibration curve is obtained by plotting on double logarithmic paper the clotting time as ordinate against factor X concentration The 100 per cent value is obtained by testing the 1 10 dilution of several normal plasmas The other values are obtained by successive dilutions of the 1 10 one The normal values lie between 14 and 17 seconds depending on the reagents used It is not desirable to use a mixture of normal plasmas because of activation Oxalated or citrated plasma can be used for the test if the calibration curves are obtained with oxalated or citrated normal plasma, respectively

Activity The factor X activity can be expressed in per cent of the normal value or in units The mean value of several normal plasmas is taken as standard By definition, 1 ml of plasma contains 100 units of factor X The per cent can be very easily converted into units and, conversely, units to per cent Per cent and units are numerically identical· 100 per cent is equal to 100 units/ml

Normal Range The normal range depends on the calibration curve. In our laboratory the normal values vary between 80 and 120 per cent In other laboratories this normal range is much broader Loeliger[4] has found variations as large as 55 to 185 per cent When the curve is very flat, the

precision of the method is less and the normal range has a tendency to become very broad. The optimal range of the method lies between 5 and 40 per cent. It is sensitive to small amounts of factor X ranging from 0.2 to 0.5 per cent.

Precautions and Sources of Error.

1. The filtration speed of bovine plasma is very important; it can vary from one plasma to another.

2. Prothrombin content and blank value must be carefully controlled If the blank values are too short, the precision of the method decreases. If the prothrombin activity is too low, prothrombin of the tested plasma can interfere.

3. A non-homogeneous Russel's viper venom-cephalin mixture cannot be used.

4 Addition of phenol to the reagents has a deleterious effect and must be strictly avoided

5. During the performance of the test, the clotting mixture is gently stirred with the platinum wire; excessive stirring disturbs the fibrin formation and the coagulation end point cannot be clearly determined.

6 In order to avoid temperature variations, the test tubes should never be taken out of the water bath This can be managed by using transparent (glass or plastic) containers

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15. Isolation and Purification of Factor X (Stuart-Prower Factor)

F. DUCKERT

This factor belongs to the group of factors which are readily adsorb on $BaSO_4$ and similar adsorbents. These factors (prothrombin, factors V IX, X and the prephase accelerator) have many chemical properties common and it is quite difficult to separate them. The first goal was obtain factor X free of other clotting factors. Chemical purification w obtained simultaneously.

Three different methods allow a complete separation of factor using the same starting material, the so-called serum concentrate The b results are obtained by means of starch gel electrophoresis[1 3] and by colur chromatography on DEAE-cellulose.[1]* The purified products are devc of other clotting factors as tested with our assays methods

Materials and Reagents

1. Serum. Whole blood is collected in small glass tubes in order to i prove the clot retraction and consequently the serum yield After coagu tion the serum is left at room temperature for 6-8 hours. The serum then separated from the clot and stored at 4°C. for at least 1 week.

2. Sodium oxalate 0 1 M $Na_2C_2O_4$ solution is used

3 Barium sulphate $BaSO_4$ for x-ray examinations from Merc Germany.

4 Sodium citrate at pH 7.8 .A 0 14 M $Na_3C_6H_5O_7 \cdot 5H_2O$ is used

5 Sodium citrate at pH 7 0 The solutions are obtained by mixing trisodium citrate solution and a citric acid solution of the same molari The concentrations used are 0 06 and 0 08 M

6 Veronal buffer, pH 9 0 7.36 Gm diethyl barbiturate Na (veror -Na) and 3 86 gm $CH_3COONa \cdot 3H_2O$ are dissolved in 1100 ml distill water The pH is adjusted with concentrated hydrochloric acid The ior strength is 0 06

7 Polyvinylpyrrolidone A 30 per cent solution of PVP 25 (Bay Leverkusen, Germany) in distilled water is used The pH of the soluti should be adjusted to neutrality

8 Hydrolyzed maize starch Maize starch is hydrolyzed according Smithies' method for the hydrolysis of potato starch Six hundred gm mai starch and 1200 ml of an acetone-HCl mixture (100 parts acetone and

* The paper electrophoresis, less suitable than the other methods, is not descrit here

part concentrated HCl) are warmed to 38°C. separately. Then starch is slowly added to the acetone-MCl mixture and gently stirred Stirring is stopped 3 minutes after complete addition of the starch. The mixture is left at 38°C. for 45 minutes Finally, 150 ml. 1 M CH_3COONa are rapidly added with stirring. The mixture is cooled and filtered on a glass filter The hydrolyzed starch is washed 3 times with 4 liters of distilled water The water is eliminated by rinsing the starch on the glass filter with acetone The resulting powder is dried in a dessicator containing P_2O_5

9 Potato starch Untreated, unhydrolyzed potato starch is used.

10. DEAE-cellulose. Type 20, 0.76 meq./Gm. from Brown & Company, Berlin, N. H, U S.A.

11. Serum concentrate Nine volumes of stored serum are mixed with 1 volume of sodium oxalate 0 1 M. Twenty Gm of $BaSO_4$ are suspended in 1 liter of oxalated serum and the suspension stirred at room temperature for 30 minutes The mixture is centrifuged and the supernatant discarded The $BaSO_4$ is resuspended in cold 0 9 per cent NaCl solution and recentrifuged.

Washing with 100 ml NaCl each time is repeated until the supernatant becomes colorless After washing, the $BaSO_4$ sediment is suspended in 60 ml. 0.14 M sodium citrate. The elution is carried out at 2°C. and pH 7.8 during 1 hour under continuous stirring When elution is completed, the $BaSO_4$ is centrifuged off and the eluate containing the clotting factors is dialyzed against cold distilled water, frequently renewed. The dialysis is continued until all ions are eliminated The solution then is concentrated 4 to 5 times by dialysis against the polyvinylpyrrolidone solution at 2°C. The concentrate of the serum clotting factors is finally frozen at −20°C. in 2 ml portions sufficient for either one electrophoresis or chromatography run

Electrophoresis on Starch Gel[3]

1 *Preparation of the gel* Eighteen Gm of hydrolyzed maize starch and 7 Gm of untreated potato starch are suspended in 220 ml veronal buffer solution. The mixture is heated directly over a flame, and the hot and viscous starch glue is poured into the cell after the filter papers have been introduced through the slits in the end walls of the cell The cell made out of Plexiglass has inner dimensions of 235 × 66 × 11 mm For the connection two sheets of Whatman chromatography paper No 1, 6 6 cm wide, are used A Plexiglass sheet, 2 mm thick and 60 mm long, is dipped in the glue to form a trench for the application of the sample 5 cm from the cathodic end of the cell which is immediately covered with 1 cm thick Plexiglass sheets, one for the cathodic and one for the anodic side of the start line trench. By pressing the covers, the excess of starch is eliminated

and no air bubbles are left in the cell When the gel begins to cool, a little air is allowed to enter the cell through 1 mm. bore holes in each corner of the cell.

2. *Performance of electrophoresis*· When the gel is cold, covers are fixed with screw clamps. The Plexiglass sheet is gently lifted out, 1.5 ml. serum concentrate is introduced into the start trench and the trench is covered with another 2 mm. thick plexiglass sheet, which is held firmly by the two covers. The starch gel cell is placed between the two buffer cells, containing 1 liter veronal buffer pH 9.0, 1 cm. away from each compartment on either end and supported underneath. The bottom of the starch cell should be slightly lower than the level of the buffer solution in both compartments. The latter measure prevents draining of the start line trench and drying of the gel Filter papers are moistened, dipped into the buffer and the electrophoresis started

Electrophoresis is performed in a cold room at 2°C at 130 V and 11-20 ma for 21 hours Best results have been obtained when, prior to the electrophoresis, another blank electrophoresis with buffer instead of serum concentrate had been performed in reverse direction and for 15 hours only, so that the cathodic buffer reached pH 11 35, whereas in the anodic compartment pH was diminished to 7.70.

3. *Recovery of the clotting factors*· After electrophoresis the covers are removed and the gel is cut into 4 mm wide strips The strips are frozen at −30°C. for 1 hour, then thawed in an incubator at 37°C The strips are centrifuged separately in glass tubes fitted with polyvinyl "mushrooms" for 10 minutes at 700 g [2] It is possible to squeeze out half of the starch gel liquid, at least 1 5 ml per fraction The fractions can be stored at −20°C. Factor X migrates faster than the other factors and the main activity is found in the fractions around 11 to 12 cm from the start line

4 *General observations.*

a. The serum concentrate should contain a high factor X activity, at least 4000 units/ml or 40 times more than plasma
b. The separation capacity of the gel varies very much when different batches of hydrolyzed maize starch are used The original Smithies' hydrolized starch has much better mechanical properties than our maize starch, but a separation with Smithies' starch is not possible
c The recovery of factor X activity varies between 30 and 50 per cent.
d. The best factor X fractions are 2300 times purer than plasma; 100 ml of normal plasma do not contain more than 2 to 3 mg. of factor X.

part concentrated HCl) are warmed to 38°C. separately. Then starch is slowly added to the acetone-HCl mixture and gently stirred. Stirring is stopped 3 minutes after complete addition of the starch. The mixture is left at 38°C. for 45 minutes. Finally, 150 ml 1 M CH_3COONa are rapidly added with stirring The mixture is cooled and filtered on a glass filter. The hydrolyzed starch is washed 3 times with 4 liters of distilled water. The water is eliminated by rinsing the starch on the glass filter with acetone The resulting powder is dried in a dessicator containing P_2O_5.

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reproducibility of the methods is quite good except when the serum concentrate contains more than a trace of citric ions.

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16. Quantitative Determination of Autoprothrombin C

E. R. COLE, E. MARCINIAK and W. H. SEEGERS

Autoprothrombin C is derived from prothrombin, but is not found in serum because of inactivation by antithrombin III The activity which is therefore usually measured is that which generates in the absence of antithrombin. Another interfering substance may be thrombin, and as a consequence we describe two procedures One of these is applicable only when there is no thrombin present whereas the other is quantitative even in the presence of thrombin.

Materials

Purified prothrombin Prothrombin was purified from bovine plasma as previously described by Seegers [1]

Autoprothrombin C A convenient way to obtain the enzyme is to follow the thrombin purification procedure of Seegers, Levine and Shepard [2] The autoprothrombin C comes from the column before the thrombin. In our work the enzyme is taken up in 50 per cent glycerol solution and stored in the freezer Around pH 7.0, autoprothrombin C is stable under these conditions

Lipid suspension This material is no different from crude cephalin used by other authors many times (see page 301).

Serum Ac-globulin Barium carbonate (200 mg /5 ml) is added to bovine serum and removed by centrifugation This was frozen and before use diluted 50 times with saline

Chromatography on DEAE-Cellulose Column[1]

1. *Preparation of the column.* 4 Gm. of DEAE-cellulose are washed several times with 50 ml. 0.9 per cent NaCl and then twice with distilled water, 200 ml each time. The DEAE-cellulose is suspended in 100 ml distilled water. The homogenous suspension is poured into a glass column 28 cm. long and 1 cm. wide (internal diameter) which is first lightly plugged with a small piece of cotton wool, just using water. The DEAE-cellulose is slowly added. The sediment is occasionally packed down with air pressure. The final height of the DEAE-cellulose column should be 20 cm and the flow rate at room temperature 20-25 seconds per drop This column should stay for at least 24 hours at 2°C. before use.

2. Chromatogram. The water in the column is gently pipetted off and replaced by the serum concentrate Two ml. serum concentrate are introduced slowly into the column. The concentrate is adsorbed in 1½ hours on the cellulose When adsorption is completed the column is filled with 0 06 M sodium citrate at pH 7.0, and connected with a reservoir containing the same solution. Fractions of 3 ml. each are recovered The flow rate is adjusted at 46 seconds per drop After collection of 15 fractions the solution on top of the cellulose is removed and replaced by 0.08 M sodium citrate at pH 7 0 The flow rate is maintained constant The factor X activity reaches its maximum in fractions 22 to 26.

3 *General observations*

a. The serum concentrate should contain about 5 mg /ml protein and at least 6000 units/ml of factor X

b. The first run never gives good separation The column may be washed and used 12 to 15 times

c The recovery of factor X activity varies between 40 per cent and 50 per cent

d The best fractions are 2500 times purer than plasma. The degree of purity is similar to the one obtained by electrophoresis. The concentration of factor X in 100 ml of normal human plasma does not exceed 2 to 3 mg

The chromatographic fractions do not contain other clotting factors; at least it is impossible with the present methods to detect any activity

Conclusion The purity of factor X obtained with either starch gel electrophoresis or chromatography on DEAE-cellulose is of the same degree. The chromatography has the advantage of separating factor VII which is eluted in the first fractions, and also the prephase accelerator when the elution is carried out at pH 5 8.[1] The fractions obtained by both methods are quite stable at −20°C and may be stored for a few months. The

reproducibility of the methods is quite good except when the serum concentrate contains more than a trace of citric ions.

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Materials

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Autoprothrombin C A convenient way to obtain the enzyme is to follow the thrombin purification procedure of Seegers, Levine and Shepard [2] The autoprothrombin C comes from the column before the thrombin. In our work the enzyme is taken up in 50 per cent glycerol solution and stored in the freezer Around pH 7.0, autoprothrombin C is stable under these conditions.

Lipid suspension This material is no different from crude cephalin used by other authors many times (see page 301)

Serum Ac-globulin Barium carbonate (200 mg /5 ml.) is added to bovine serum and removed by centrifugation This was frozen and before use diluted 50 times with saline.

Standard bovine plasma: This was the standard plasma used by Ware and Seegers[3] Bovine blood was mixed with 1.85 per cent potassium oxalate in the ratio of 9 parts of blood to 1 part anticoagulant. The plasma was obtained by centrifugation and stored in a deep freezer.

Procedure

Autoprothrombin C assay with purified prothrombin as substrate: In the presence of lipids, serum Ac-globulin and an optimum concentration of calcium ion, the extent to which prothrombin is activated to thrombin is dependent on the quantity of autoprothrombin C. To perform an analysis a reaction mixture of the following composition is made:

Purified prothrombin (4,500 U/ml)	0 4 ml
0 4 per cent suspension crude lipid in saline	0 2 ml.
Serum Ac-globulin	0.3 ml
$CaCl_2$ (0 32 M in pH 7 2 imidazole buffer)	0.1 ml.
Autoprothrombin C in saline	0.2 ml

Prothrombin is the last component of the reaction mixture to be added, and the generation of thrombin in this mixture is followed by removing a portion of the mixture at various times and assaying for thrombin.[4] In almost all cases, activation of prothrombin to thrombin is complete in 12 minutes Incubation of the reaction mixture is carried out at 28°C. and in paraffin-coated tubes to prevent the adsorption of prothrombin and thrombin on glass The amount of thrombin generated is expressed as thrombin units per ml of reaction mixture and depends on the quantity of autoprothrombin C in the reaction mixture (fig 1) The amount put in is regulated so that about 300-700 units of thrombin are produced This we consider the most accurate range The number of autoprothrombin C units is determined from a standard set of curves such as figure 1, and the appropriate dilution and correction factors are applied in order to express the autoprothrombin C concentration of the solution being assayed

As an example of the calculations involved in the quantitative assay, an autoprothrombin C sample was diluted in saline 40 times and 0 2 ml of this dilution was used in the reaction mixture The generation of thrombin followed a curve like that obtained with 4 units of standard autoprothrombin C in figure 1 The total dilution of the autoprothrombin C was 40 times in saline and 6 times in the reaction mixture So the concentration of autoprothrombin in the sample was $40 \times 6 \times 4 = 960$ units per ml

With samples of autoprothrombin C that contain thrombin, the amount of thrombin should be determined by thrombin assay,[4] and, by applying the dilution factors, the number of thrombin units per ml of reaction mixture contributed by the autoprothrombin C sample can be calculated and

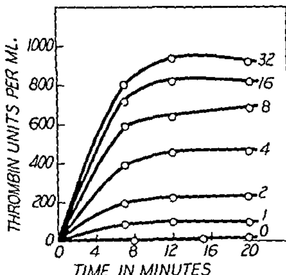


FIG 1—Quantitative yields of thrombin with various amounts of autoprothrombin C placed in the reaction mixture described in the text. The substrate was purified prothrombin The numbers with each curve represent the units of autoprothrombin C in the reaction mixture

tracted from the total thrombin titer of the reaction mixture. Otherwise, the calculations involved in measuring autoprothrombin C activity are the same as in the example given above

Autoprothrombin C assay with plasma as substrate Autoprothrombin C dilutions are handled in paraffin-coated glassware Thrombin invalidates the work Two-tenths (0 2) ml of standard plasma is pipetted into a 10 × 75 mm test tube and placed in a 37° C. water bath. Two-tenths (0 2) ml of 0 1 per cent lipid suspension in saline is added and the tube is shaken slightly to mix the plasma and lipid The tube is replaced in the water bath Immediately one adds 0 2 ml of autoprothrombin C in 0 025 M $CaCl_2$ (pH 7.0). The tube is tilted gently back and forth in the water bath, and the appearance of a solid clot is taken as the end point The time in seconds from the addition of authoprothrombin C solution to clot formation is the clotting time The autoprothrombin C solution being assayed should be diluted so that a clotting time of 12-16 seconds is obtained. The total dilution is multiplied by the unit conversion factor which depends on the clotting time (table 1) The value obtained is expressed as units of autoprothrombin C per ml of the original autoprothrombin solution On the basis of the work described above, a unit of autoprothrombin C can be

Standard bovine plasma: This was the standard plasma used by Ware and Seegers.[3] Bovine blood was mixed with 1.85 per cent potassium oxalate in the ratio of 9 parts of blood to 1 part anticoagulant. The plasma was obtained by centrifugation and stored in a deep freezer.

Procedure

Autoprothrombin C assay with purified prothrombin as substrate. In the presence of lipids, serum Ac-globulin and an optimum concentration of calcium ion, the extent to which prothrombin is activated to thrombin is dependent on the quantity of autoprothrombin C. To perform an analysis a reaction mixture of the following composition is made.

Purified prothrombin (4,500 U /ml)	0 4 ml
0 4 per cent suspension crude lipid in saline	0 2 ml
Serum Ac-globulin	0.3 ml
$CaCl_2$ (0.32 M in pH 7 2 imidazole buffer)	0 1 ml
Autoprothrombin C in saline	0 2 ml

Prothrombin is the last component of the reaction mixture to be added, and the generation of thrombin in this mixture is followed by removing a portion of the mixture at various times and assaying for thrombin.[4] In almost all cases, activation of prothrombin to thrombin is complete in 12 minutes Incubation of the reaction mixture is carried out at 28°C. and in paraffin-coated tubes to prevent the adsorption of prothrombin and thrombin on glass The amount of thrombin generated is expressed as thrombin units per ml of reaction mixture and depends on the quantity of autoprothrombin C in the reaction mixture (fig 1) The amount put in is regulated so that about 300-700 units of thrombin are produced. This we consider the most accurate range The number of autoprothrombin C units is determined from a standard set of curves such as figure 1, and the appropriate dilution and correction factors are applied in order to express the autoprothrombin C concentration of the solution being assayed

As an example of the calculations involved in the quantitative assay, an autoprothrombin C sample was diluted in saline 40 times and 0 2 ml of this dilution was used in the reaction mixture The generation of thrombin followed a curve like that obtained with 4 units of standard autoprothrombin C in figure 1 The total dilution of the autoprothrombin C was 40 times in saline and 6 times in the reaction mixture So the concentration of autoprothrombin in the sample was $40 \times 6 \times 4 = 960$ units per ml

With samples of autoprothrombin C that contain thrombin, the amount of thrombin should be determined by thrombin assay,[4] and, by applying the dilution factors, the number of thrombin units per ml of reaction mixture contributed by the autoprothrombin C sample can be calculated and sub-

17. Estimation of Hageman Factor in Plasma or Plasma Fractions (Adaptations of Methods of Margolis[1] and Rapaport[2])

O. D. RATNOFF

Principle: The preparation to be tested is incubated with kaolin to insure maximal activation of Hageman factor. The mixture is then incubated with Hageman factor-deficient plasma; in this step, any Hageman factor in the preparation presumably activates the PTA contained in the Hageman factor-deficient plasma. Finally, the mixture is recalcified and its clotting time is measured; the clotting time appears to be a function of the concentration of Hageman factor in the preparation tested. Small amounts of phospholipids are included in the kaolin mixture to accelerate the formation of the clot.

Reagents and Apparatus Required

(1) Preparation to be tested *Plasma* is prepared from blood to which one-ninth volume of 0 13 M sodium citrate solution has been added *Fractions of plasma* are dissolved or diluted in barbital-saline-citrate buffer

(2) Barbital-saline buffer 7 30 Gm of sodium chloride, 2 76 Gm. of barbital and 2 06 Gm of sodium barbital, diluted to a volume of 1 liter with distilled water

(3) Barbital-saline-citrate buffer 85 ml of barbital-saline buffer are mixed with 15 ml of 0 13 M sodium citrate

(4) Kaolin Fisher, N F, acid-washed

(5) Crude Phospholipid. "Cephalin," prepared from rabbit brain by the method of Bell and Alton (Nature 174.880, 1954) is supended at a concentration of 6 mg per ml of 0 15 M sodium chloride Aliquots of 0 1 ml are stored in 15 ml Lusteroid tubes at −25°C.

(6) Hageman factor-deficient plasma Platelet-deficient citrated plasma is prepared in silicone-coated tubes from the blood of a patient with Hageman trait and stored in silicone-coated tubes at −25°C until used The citrated plasma is obtained from venous blood to which one-ninth volume of 0 13 M sodium citrate solution has been added

(7) Calcium chloride solution· 0 04 M

Steps in the Performance of the Test

(1) On the day of the test, a tube of "cephalin" is thawed and diluted with 9 9 ml barbital saline buffer To 5 ml of this mixture, 50 mgm. of

TABLE 1: *Variations of Clotting Time with Autoprothrombin C Concentration*

Clotting Time (seconds)	Units	Clotting Time (seconds)	Units
17 0	0.92	14 4	1.03
16 8	0 92	14 2	1 04
16 6	0 93	14 0	1.05
16 4	0 93	13.8	1.06
16 2	0 94	13 6	1 08
16 0	0 95	13.4	1 09
15 8	0 96	13 2	1 11
15 6	0 97	13 0	1 13
15 4	0 98	12 8	1.16
15 2	0 99	12 6	1.18
15 0	1 00	12 4	1.23
14 8	1 01	12 2	1.27
14 6	1 02	12.0	1.30

defined as the amount which will generate 70 units of thrombin in a standardized prothrombin activation mixture.

As an example of the calculations involved, an autoprothrombin C solution was diluted 50 times in saline One (1 0) ml. of this dilution was mixed with one (1.0) ml. of 0 05 M $CaCl_2$, pH 7.0 Two-tenths (0 2) ml of the autoprothrombin C in $CaCl_2$ mixture gave a clotting time of 14 6 seconds, or a conversion factor of 1.02 (table 1). $50 \times 2 \times 3 \times 1.02 = 306$ autoprothrombin units per ml The dilutions involved were:

1. Diluted 50 times with saline.
2. Diluted 2 times with 0 05 M $CaCl_2$
3. Diluted 3 times in the clotting mixture.

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kaolin is added and mixed in a hand or mechanical homogenizer. One-tenth ml. of the suspension is pipetted into each of a series of Pyrex tubes (internal diameter 8 mm). These tubes are kept in an ice bath until needed.

(2) The plasma to be tested is diluted 10-fold in a silicone-coated tube by the addition of barbital-saline-citrate buffer, and kept in an ice bath until used. Further dilutions of the plasma are prepared as needed

(3) One-tenth ml. of diluted plasma is added with a silicone-coated pipette to 0.1 ml. of Kaolin-"cephalin." The tube is gently tapped to mix its contents and incubated at 37°C for 2 minutes. Then 0.1 ml. of Hageman factor-deficient plasma is added with a silicone-coated pipette. The tube is once again tapped and incubated at 37°C. for 8 additional minutes. Finally, 0.1 ml of 0 04 calcium chloride solution is added and a stop watch is started. After 30 seconds, the tube is tilted continuously until a clot forms The end point is sharp and usually unmistakable.

(4) Fractions of plasma are tested in a similar manner, diluting the preparation with barbital-saline-citrate in silicone-coated tubes. The greatest accuracy is obtained if fractions of plasma are tested as soon after dilution as is feasible.

Calculations: No absolute standard for the concentration of Hageman factor in plasma is as yet available. The relative concentrations of Hageman factor in different samples of plasma or in fractions of plasma are estimated in the following manner The most active sample—that is, the one which accelerates the clotting of Hageman factor-deficient plasma the most—is diluted serially with barbital-saline-citrate buffer Each dilution is tested until the clotting time exceeds that of the weakest sample under study A calibration curve is constructed on double logarithmic paper The activity of each sample is calculated by interpolation. Among 41 normal adult subjects, the concentration of Hageman factor in plasma ranged from 38 to 152 per cent of the concentration in a pool of these plasmas. In three-fourths of the subjects, the concentration of Hageman factor was between 76 and 112 per cent of the pool. No differences were found between males and females or white or Negro subjects [8]

Precautions and Sources of Error. The assay for Hageman factor is still primitive and modifications to increase its accuracy can be anticipated. Meticulous care in pipetting and attention to the order and timing of additions of the various reagents are necessary to insure accuracy The presence of activated PTA in the sample to be tested vitiates the assay This is to be expected, since one function of Hageman factor appears to be the activation of PTA.

g. Veronal buffered oxalated saline solution (VBOS):

Veronal-buffered-isotonic-saline (VBIS)

0.1 M sodium diethylbarbiturate	200 ml.
0.1 M HCl	144 ml.
0 15 M NaCl	656 ml

Veronal-buffered-oxalated-saline (VBOS)

0.1 M sodium (or potassium) oxalate	150 ml.
VBIS	850 ml.

h. *Thromboplastin:* Human brain,* preferably small and if possible intact, should be used as fresh as possible. Pia mater and blood vessels are removed carefully and as completely as possible. The brain is washed first with tap water, followed by saline, dried on filter paper to remove as much water as possible and then cut into small portions The brain is next extracted with C.P. acetone, 3 times its volume, using small batches in a Waring blender for 15 seconds. The mixture of brain and acetone is then filtered through filtered paper and the acetone discarded The residue is gently stirred with 5 volumes acetone for 5 minutes and then put into the Waring blender for 10 seconds This operation can be repeated until the brain residue gives to the hand a feeling of smooth dryness. If more than three extractions are necessary, the brain should not be put again into the blender but just washed in acetone *Precaution·* No smoking and no fluorescent lighting during acetone extraction As soon as the extraction is completed, the powder is spread on a large sheet of non-absorbent paper and left overnight at room temperature The residue is then ground in a mortar and passed through a strainer The gross residue is now discarded. The strained material thus obtained is then stored in a vacuum desiccator at 4°C Full potency is maintained for at least 1 year

Thromboplastin extracts are made by extracting the brain powder with saline as follows 5.0 Gm of the powder are mixed with 100 ml of physiologic saline, incubated at 50°C for 15 minutes, being inverted twice every 2 minutes. It is then centrifuged for 5 minutes at 2000 rpm The supernatant is filtered through two thicknesses of gauze. The extract is subdivided in separate test tubes, the volume in each tube depending upon estimates of anticipated need of thromboplastin for a day's work (once thawed, the thromboplastin solution cannot be frozen again). The tubes are kept frozen at −15 to −20°C. No more thromboplastin solution should be made than the anticipated need for 1 week's work, in order that it should give a Quick prothrombin time no longer than 14 seconds

* Brain preparations from other species may be used rabbit brain prepared according to the method of Quick[3] or commercial brain thromboplastin preparations In *all* instances, the potency of the thromboplastin extract being used must be ascertained on a pool of plasma from at least five normal subjects

CHAPTER VII

THROMBIN AND PRECURSORS

1. One-Stage Specific Determination of Factor II (Prothrombin)*

L. PECHET

Object of the Method· The one-stage prothrombin test of Quick is sensitive to at least five blood coagulation factors: I (fibrinogen), II (prothrombin), V (Ac-globulin), VII (proconvertin) and X (Stuart) and therefore lacks specificity. When precise information is desired as to the exact concentration of any individual factor, the other four have to be supplied in optimal amounts. This is accomplished in the case of factor II either by a two-stage procedure, described in this chapter (p 159), or by a simpler, one-stage method to be considered here

Principle: The validity and the specificity of the procedure is predicated upon the assurance that under the conditions of the test, the only variable which will determine the prothrombin time is the concentration of factor II A modification of the Owren[1] method seems to satisfy these criteria. (1) Factors I and V are provided by bovine plasma from which Factor(s) II (and VII) have been removed by absorption on $BaSO_4$ Human serum is also incorporated in order to restore the factor VII removed by $BaSO_4$, without at the same time restoring factor II The procedure described is suited both for the determination of plasma and serum factor II, thus being an equally useful technic for the prothrombin consumption test.

Reagents and Apparatus

a Water bath thermoregulated to maintain a temperature of 37°C.

b Test tube rack and glass tubes, 10 × 75 mm

c. Pipettes, 1.0 ml. calibrated to 0 01 ml. subdivision. Blowout pipettes calibrated to deliver 0.10 ml

d Stop watch.

e. 0 04 M $CaCl_2$ solution.

f 0.1 M sodium oxalate and 0 15 M potassium oxalate solutions.

* The present text is based partially on the method described by Dr Benjamin Alexander in Chapter VII of the first edition of this book

thrombin concentrations below 5 per cent of that in normal plasma (fig. 1). The prothrombin content of a test material appropriately diluted with VBOS is computed by interpolating the observed clotting time on the standardization curve. Such a curve must be derived on each day the test is performed. In 48 normal subjects, the range for factor II found in our laboratory in 1 day was 66 to 145 per cent (± 2 standard deviations on a logarithmic scale), with a mean of 98 per cent. The accuracy of the method for an individual test is approximately ± 5 per cent.

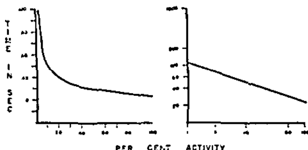


FIG. 1—Typical standardization curve, correlating clotting time with prothrombin concentration, used in one-stage method for determining prothrombin specifically

Precautions, Sources of Error and Limitation Since most, if not all of the factors other than prothrombin which affect the prothrombin time are adequately controlled in this method, many of the precautions, sources of error and limitations pertinent to the procedure for measuring the Quick one-stage prothrombic activity do not apply here Thus, determinations can be made on aged plasma, and consideration need not be given to the deterioration of factor V which, in this test, is provided by the $BaSO_4$ bovine plasma. Similarly, less concern need be given to the technic of obtaining the blood sample for activation of factor VII will not affect the test (this factor is provided by the human serum) This does not imply, however, that carelessness can be condoned, since poor technic and delay in mixing the blood with anticoagulant may result in substantial prothrombin losses, causing erroneously low values

Each determination is performed in duplicate, and the clotting times must agree within 1 second Certain precautions regarding the procurement of bovine plasma are noteworthy. The blood is obtained from the major vessels at the slaughterhouse when the animal is sacrificed From a bucket of blood rapidly obtained, a measured quantity is quickly poured into a receptacle containing anticoagulant, and the mixture is thoroughly stirred Blood contaminated by esophageal and rumen contents, consequent to overzealous slitting of the throat, should be discarded

i. *Thromboplastin-$CaCl_2$ mixture:* Equal parts of freshly thawed human brain thromboplastin extract (prepared as described above) and 0.04 M $CaCl_2$ solution.

j. *$BaSO_4$ adsorbed bovine plasma:* Beef blood is collected in 0.15 M potassium oxalate (1 volume to 9 volumes of blood). The plasma is treated with $BaSO_4$* for 30 minutes with constant stirring. Then the mixture is centrifuged at 3000 rpm for 30 minutes, the supernatant carefully separated from the sedimented $BaSO_4$, which is discarded. The barium sulfate-adsorbed plasma is the source of factor V in the system.

k. *Human oxalated serum.* Normal blood is added to thromboplastin (100 ml. blood to 3 ml thromboplastin) and well mixed to induce rapid and complete coagulation, the clotted blood is allowed to stand at room temperature for 24 hours, the serum is separated by centrifugation, then oxalated (1 volume of 0.1 M sodium oxalate to 4 volumes serum), followed by centrifugation to eliminate the precipitate.

The $BaSO_4$-adsorbed bovine plasma and oxalated human serum are mixed in equal proportions, and divided into aliquots each of sufficient volume for 1 day's determinations. Stored in the frozen state (−15 to −20°C), the mixture is stable for at least 6 months. Repeated thawing and freezing causes deterioration; thus a tube containing one aliquot is thawed, used for 1 day's experiments, and then discarded.

Performance of Test. To 0.1 ml of the substrate (bovine $BaSO_4$ plasma-serum mixture), 0.1 ml of normal human plasma diluted tenfold with VBOS† is added. This dilution is necessary because of the very short clotting times obtained in the presence of even small amounts of prothrombin under the conditions of this test. The mixture is allowed to attain the water bath temperature, and 0.2 ml of the thromboplastin-calcium mixture are blown into the tube. A stop watch is started simultaneously. The tube is tilted gently starting 10 seconds before the expected clotting time (learned by experience) until a solid gel is observed. The value thus obtained with normal plasma is taken to be equivalent to 100 per cent prothrombin concentration. Further serial subdilutions with VBOS of the original 1 to 10 dilution of normal plasma will give progressively longer clotting times which reflect the correspondingly lower concentrations of prothrombin. The standardization curve is thus obtained by plotting the observed clotting times against the per cent prothrombin. When plotted log/log, a linear relationship is obtained except (occasionally) for pro-

* It should be noted that $BaSO_4$ from different chemical houses may vary in its affinity for prothrombin. We have found that the Baker Co. material has the greatest affinity. One hundred mg $BaSO_4$ are used for each milligram of plasma.

† Such dilution has the added advantage of obviating to a large extent, if not entirely, the possible presence of heparin or other anticoagulants in the test material.

This is continued until the preparation becomes granular. The material is filtered by suction and dried at room temperature on a large glass surface with continual turning over until the odor of acetone is no longer evident.

Steps in the Procedure: Nine volumes of blood obtained by venepuncture are mixed with 1 volume of 3.8 per cent sodium citrate or, alternately, 4 9 volumes of blood are mixed with 0.1 volume of 19 per cent sodium citrate. The plasma is separated from the cellular elements by centrifugation at 2000 g for 15 minutes at 4°C. The plasma is removed by means of a pipette with a rubber bulb.

The prothrombin time is determined by transferring 0 1 ml of plasma to a small Pyrex test tube which is placed in the water bath at 37°C. After 1 minute, 0 1 ml of thromboplastin solution (previously kept at 37°C for 5 to 10 minutes) is added, 0 1 ml. of 0 02 M calcium chloride is blown forcibly into the mixture and the stop watch started simultaneously The tube is put in the water bath and shaken lightly A few seconds before the expected clotting time, the tube is held toward a distant source of light so that one can see through the tube The tube is tilted very gently to permit detecting the incipient web of fibrin, which is the end point

When the prothrombin time is greatly prolonged, it is convenient to replace the test tube in the water bath for a few seconds at intervals before the end point is reached

Calculation: The results of the test are expressed as per cent of prothrombin activity. For each lot of frozen thromboplastin a standard curve for prothrombin activity is made. Normal plasma is assayed for prothrombin content by the two-stage method (p 159). The prothrombin time of this plasma is determined Using log-log graph paper, the clotting time (ordinate) is plotted against the per cent prothrombin concentration (abscissa) as determined by the two-stage assay The plasma and $CaCl_2$ solutions are diluted with BPSS to 75 per cent, 50 per cent and 25 per cent concentration and tested in the one-stage system The plasma dilutions should be tested immediately after preparation Plotting the clotting time against the corresponding per cent prothrombin concentration results in a straight line which can be extrapolated to ten per cent The per cent prothrombin activity for an unknown plasma is read directly from the standard curve

Sources of Error. Oxalate can be used as anticoagulant for the blood collection providing special care is exercised in handling the blood and plasma After a few hours at 37°C or room temperature, there is rapid loss of factor V activity in oxalated plasma which results in lengthening of the prothrombin time of the plasma Oxalated plasma cannot be stored at 4°C. overnight for this test.

ACKNOWLEDGMENT

The author wishes to acknowledge the help of Miss Frances Cochios in preparing this manuscript.

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2. Estimation of Prothrombin (One-Stage Method of Quick)

Described by L. M. TOCANTINS

Apparatus and Reagents

(1) Water bath—37°C.

(2) A stop watch

(3) Pipettes 1 ml serologic, graduated in tenths.

(4) Sodium citrate, 3 8 per cent 3 8 Gm. sodium citrate dissolved in 100 ml of distilled water or sodium citrate 19 per cent· 19 Gm. sodium citrate dissolved in 100 ml distilled water

(5) Calcium chloride 0 02 M

(6) Test tubes pyrex (12 × 75 mm.).

(7) Thromboplastin solution—3.0 Gm of acetone-dried brain powder is placed in an Erlenmayer flask, 50 ml of 0.85 per cent sodium chloride solution is added, and the contents mixed The mixture is incubated for 30 minutes at 48°C with gentle swirling every 10 minutes. The extraction is cooled to 4°C and allowed to stand 1 hour at 4°C The suspension is centrifuged at 1200 g for 10 minutes at 4°C. The supernatant is carefully decanted to avoid including gross particles from the sediment The supernatant is tested for potency and, if satisfactory, is quick-frozen in a dry-ice-acetone bath in desired aliquots The aliquots are thawed at 37°C for use

(8) *Preparation of acetone dried powder* The brain is stripped of meninges and blood vessels, cut into small pieces and then triturated with acetone in a porcelain mortar or Waring blender The acetone is poured off, the brain is covered with fresh acetone, and the trituration repeated.

3. Assay of Prothrombin: One-Stage Method Using Dilute Plasma (Method of Campbell, Smith, Roberts and Link)

Described by R. R. HOLBURN*

Object of the Method: Modifications introduced into the Quick one-stage prothrombin time designed to increase reproducibility, and decrease the minimum detectable change in prothrombin

Principle The determination, under standard conditions, of the time required for the clotting of diluted recalcified plasma in the presence of an excess of thromboplastin.

Apparatus and Reagents

Sodium oxalate: 1 34 Gm of sodium oxalate C P dissolved in 100 ml of distilled water. Solution is 0.1 M.

Calcium chloride: 0 222 Gm anhydrous calcium chloride dissolved in 100 ml of distilled water. Solution is 0 02 M

Thromboplastin. Dry rabbit brain tissue is prepared as follows. a rabbit is decapitated by a sharp pair of tin snips The brain tissue and available medulla are removed. The free pia mater is removed under running water The tissue is ground to a fine paste in a mortar, spread out on a watch glass, and dried in a vacuum dessicator containing calcium chloride. The air is removed until a large foamy mass is obtained, whereupon the dessicator is maintained at 38–40°C for 24 hours These operations are executed as rapidly as possible The dry tissue is then broken up into a fine powder and stored at 0°C

Preparation of emulsion To 0 1 Gm of the dried tissue is added 5 0 ml. of 0 85 per cent NaCl The suspension is stirred and heated at 54–55°C in a water bath for 10 minutes to destroy prothrombin activity The suspension is cooled to 25–26°C; 5 0 ml of 0 025 M calcium chloride is added to the suspension After being stirred for 4 minutes, the mixture is centrifuged for 4 minutes at 1700 rpm The clear or slightly turbid supernatant is carefully removed by pipette

Plasma The blood syringe is rinsed with 0 1 M oxalate solution and 0 2 ml of oxalate is drawn into the cylinder From the dilated vein 1 8 ml blood is drawn and mixed quickly by rotating the syringe The oxalated blood is gently forced from the syringe into a 75 × 10 mm test tube The

* From Journal of Biological Chemistry 138 1, 1941

Preparations of thromboplastin may vary in their sensitivity to depressions in other clotting factors. Human thromboplastin often reflects a greater sensitivity to decreases in factors V, VII, IX and X in human plasma than thromboplastins from other species. The use of thromboplastin from other tissue sources such as lung, which have a greater contamination with blood, will result in preparations less sensitive to the concentration of factors VII, IX and X.

In preparing the standard curve, concentrations of normal plasma below 25 per cent should not be used since the loss of fibrinogen and factor V activity will increase the clotting time and cause the curve to deviate from linearity. If it is necessary to standardize as low as 5 to 10 per cent, the plasma may be diluted with 0.5 per cent fibrinogen solution and $BaCO_3$-adsorbed beef serum diluted 1:75 to supply factor V.

Because of the exponential relationship between clotting time and prothrombin concentration, prothrombin activity cannot be calculated by dividing the prothrombin time of normal plasma by that of the unknown

If the hematocrit of the blood sample varies from normal, the concentration of anticoagulant in the plasma will be changed. The optimum calcium chloride concentration must be determined for these samples before the test is performed. This is especially true when the hematocrit is high as in polycythemia.

Reference

Quick, A J. The determination of prothrombin by the method of Quick. *In:* Transactions of the 1st Conference on Blood Clotting and Allied Problems of the Josiah Macy, Jr. Foundation, 1948, p 170

ferential method, these variations in absolute values will not influence the reliability of the assay. If the minimum values for the clotting times are sought, they may be realized by making serial dilutions of the calcium chloride solution.

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4. Estimation of the Blood Prothrombin by the Bedside Method

L. B. JAQUES

This consists of the determination of the clotting power of the blood to which an excessive amount of thromboplastin has been added. The object of the bedside prothrombin test is to provide a simple rapid method for the determination of prothrombin times. It has been called the accelerated or activated clotting time of the blood. It is particularly useful with small animals.

Reagents

Thromboplastin: For the Smith bedside method, the thromboplastin is prepared from fresh perfused ox or rabbit lung (see pp. 161, 201).

For the Schwager-Jaques method, rabbit brain thromboplastin is used, prepared as described (p. 148). The thromboplastin may be placed in wide-bore capillary tubes (over 2 mm. in diameter and 4 inches long) with about 2 inches being drawn into each tube. The tubes are sealed in a flame and immediately frozen in the freezing compartment of a refrigerator and kept frozen until used. This thromboplastin extract will remain unchanged in activity for as long as 6 months, when preserved in this manner. Before use, the tube is removed from the refrigerator and warmed in the hand, if required for immediate use.

Procedure

Smith bedside method:[1] One-tenth ml. thromboplastin is placed in a small tube (75 × 10 mm.) which has been marked at 1.0 ml. Freshly

formed elements are removed by centrifugation at 1700 rpm The plasma is removed by pipette.

Assay and Steps in Procedure: Into two 75 × 10 mm. test tubes, 0.1 ml of plasma is added with a serologic pipette graduated to 0.001 ml. To one tube is added 0.7 ml. of 0.85 per cent NaCl solution to give a concentration of 12.5 per cent. To the other is added 1.1 ml. of saline to give a concentration of 8.34 per cent. The diluted plasmas are mixed thoroughly and placed in a 37°C. water bath. Into 100 × 12 mm. tubes 0.2 ml. of the thromboplastin-calcium chloride solution is transferred, using a 0.2 ml micro blood sugar pipette. These tubes are placed in the water bath. When the tube contents have reached the bath temperature, 0.1 ml. of the diluted plasma is added to the tube containing thromboplastin-calcium chloride solution The diluted plasma is blown from the pipette and the stop watch started simultaneously The tube is tapped sharply to mix the contents A small stirrer made of #2 nichrome wire is introduced into the tube; the stirrer paddle sweeps the test tube from side to side twice per second. The end-point is that point when the fibrin clot is sufficiently stable to be drawn to one side by the stirrer. The variations between duplicate determinations on these diluted plasmas will be one second or less Dilutions of the test plasma are chosen so that the clotting time will fall between that of the two dilutions of the normal plasma A concentration higher than 25 per cent should not be used; if necessary, the normal plasma may be further diluted to provide an additional range.

Calculation. A dilution curve of the standard plasma is made, the points between 5 and 20 per cent falling on a straight line on log-log paper. Log of the plasma concentration is the ordinate, log of clotting time is the abscissa. The clotting time of the test plasma is interpolated on the standard chart and the log of the ordinate read The antilog of the ordinate is calculated, this is the concentration of normal plasma which will give a clotting time equal to that of whatever concentration of test plasma was used Therefore, the relative clotting index or the ratio of the concentration of normal plasma to the concentration of the test plasma is equal to the antilog of the ordinate divided by the per cent of the test plasma that was assayed

Precautions and Sources of Error (1) Dilution curves of all individuals of any species will not be alike, therefore, a normal dilution curve should be set up for each individual for reproducible results. (2) By mixing the thromboplastin and calcium together and placing in an empty tube, the [illegible] of stock reagents is [illegible] (3) The variations [illegible] values [illegible] a dif-

ferential method, these variations in absolute values will not influence the reliability of the assay. If the minimum values for the clotting times are sought, they may be realized by making serial dilutions of the calcium chloride solution.

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4. Estimation of the Blood Prothrombin by the Bedside Method

L. B. JAQUES

This consists of the determination of the clotting power of the blood to which an excessive amount of thromboplastin has been added. The object of the bedside prothrombin test is to provide a simple rapid method for the determination of prothrombin times It has been called the accelerated or activated clotting time of the blood It is particularly useful with small animals.

Reagents

Thromboplastin: For the Smith bedside method, the thromboplastin is prepared from fresh perfused ox or rabbit lung (see pp 161, 201).

For the Schwager-Jaques method, rabbit brain thromboplastin is used, prepared as described (p 148) The thromboplastin may be placed in wide-bore capillary tubes (over 2 mm in diameter and 4 inches long) with about 2 inches being drawn into each tube The tubes are sealed in a flame and immediately frozen in the freezing compartment of a refrigerator and kept frozen until used This thromboplastin extract will remain unchanged in activity for as long as 6 months, when preserved in this manner Before use, the tube is removed from the refrigerator and warmed in the hand, if required for immediate use

Procedure

Smith bedside method[1] One-tenth ml thromboplastin is placed in a small tube (75 × 10 mm) which has been marked at 1 0 ml Freshly

drawn blood is placed in the tube up to the 1 ml. mark. The tube is inverted once for complete mixing of the blood and thromboplastin, and is then tilted every second or two to observe clotting.

Schwager, Jaques method.[2] One-tenth ml. of thromboplastin extract of the contents of one capillary tube are expelled into the center of a watch-glass. Venous blood is drawn into a syringe using a 20 gauge needle. Four drops of blood are added from the needle to the thromboplastin on the watch glass. The needle is removed from the syringe. The watch glass is held on the tips of the fingers and its contents mixed immediately with the needle. The needle is passed gently through the mixture until it clots. The end point is sharp The time elapsed from addition of the first of the four drops of blood to the end point is the activated clotting time. In normal blood the time by this method is 23 to 25 seconds. Range 2 s.d. as per cent of mean = 5.5 per cent. This mean should be calculated from results of the test on at least 10 normal adult men and women.

The range is expressed best in terms of twice the standard deviation (s d.) as a per cent of the mean Thus, with a mean activated clotting time of 23.5 seconds, twice the standard deviation was found to be ± 1 1 seconds, which is 5.1 per cent of the mean. With a mean time of 64 2 seconds, twice the standard deviation was 3 7 seconds, which is 5.5 per cent of the mean Therefore, the range expressed as twice the standard deviation is about 5 per cent of the mean Results are reported in seconds or in per cent of normal as in the Smith method, already described

Advantages· (a) A close correlation exists between the activated clotting time of the blood as performed with this technic, and its prothrombin content. Therefore, an immediate rapid estimate of the blood prothrombin is possible (b) The concentration of calcium and other blood constituents used in the test is close to that of the subject's blood

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5. The Thrombotest Method

P. A. OWREN

Object of the test: To control anticoagulant therapy.

Principle: In a coagulation system, all coagulation factors are held constant except the four to be determined: factor II (prothrombin), factor VII (proconvertin), factor IX (PTC, Christmas factor, antihemophilic B factor) and factor X (Stuart-Prower factor). The thrombotest reagent is specifically deficient in these four factors, and the coagulation time is therefore exclusively dependent on the concentration of these factors present in the blood sample to be tested. The intrinsic coagulation system has been accelerated by the introduction of an active cephalin preparation, whereas the extrinsic system has been retarded by the introduction of a thromboplastin of low activity. Thus, both coagulation systems take part in coagulation and the method also signalizes such disproportionate and excessive reductions of factor IX which may give rise to bleeding during anticoagulant therapy. Further, a thromboplastin of high sensitivity to factor X, in addition to VII and II, has been chosen, whereby the method has become especially sensitive for disclosing deficient factor X activity, which is the most common cause of bleeding during treatment with oral anticoagulants.

Reagents and Apparatus.

(1) *Thrombotest reagent* * This contains (a) thromboplastin of low reactivity but high sensitivity to factors X and VII, prepared from animal brain; (b) crude cephalin of high activity, (c) adsorbed bovine plasma, and (d) calcium. It is supplied as a freeze-dried substance in vacuum-sealed ampoules of two different sizes, one for 40 and the other for 8 tests

(2) Water bath at 37°C

(3) Small glass test tubes about 6 cm long with an internal diameter of about 8 mm

(4) Pipettes for measuring the solvent (11 ml or 2 2 ml), dissolved reagent (0 25 ml), and blood (0 05 ml) Short pipettes are recommended they are easier to fill and they empty more cleanly and quickly

(5) A stop watch

(6) a for capillary blood a sharp, pointed blade or lancet
 b for venous blood: a 2 ml or 5 ml syringe, or plastic (polythene or polystyrene) collecting tubes marked for 2 ml or 5 ml volumes.

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*Manufactured by Nyegaard & Co , Oslo, Norway

Steps in the Performance of Test: The test may be carried out with capillary blood or venous blood, but the principle is the same for both methods:

Method for capillary blood

Preparation of reagent:

(1) Remove pointed top of ampoule with forceps, then file and break off in usual place.

(2) Add 11 ml. of lukewarm (20-37°C.) distilled water to large ampoules and 2.2 ml. to small ones Shake vigorously for a short period of time and reagent is ready for use after 2-3 minutes.

(3) Pipette off 0 25 ml. of reagent into small test tubes which are placed in water bath at 37°C. and left there between 3-30 minutes.

Collection of blood.

(4) Collect blood sample from ear lobe or finger pad by making an incision and measuring off 0.05 ml. blood from first drops as fast as possible into pipette.

Clotting time determination.

(5) Immediately blow blood sample from pipette into reagent and start stop watch simultaneously.

(6) Mix blood and reagent by flicking once and leave tube in water bath for 30 seconds if normal blood, and for 50 seconds if blood from patients on anticoagulant therapy.

(7) At short intervals thereafter, remove tube from water bath and tilt to observe and record moment of coagulation

Method for venous blood

Preparation of reagent

(1) Proceed as for capillary blood, steps 1, 2 and 3, but use a 3.2 mM solution of calcium chloride as a solvent

Collection of blood

(2) Measure 0 2 ml. (or 0 5 ml.) of 3 13 per cent sodium citrate (sodium citrate $2H_2O$) solution in a 2 ml (or 5 ml) syringe and, after venepuncture, fill syringe with blood to a volume of 2 ml (or 5 ml.). Transfer blood immediately to a "non-activating" tube (plastic or siliconized glass). Alternatively, measure the citrate solution into plastic collecting tubes marked for 2 ml (or 5 ml) volumes, and allow blood to flow directly from needle into tube without using syringe

(3) Measure off 0 05 ml citrated blood into pipette.

Clotting time determination

(4) Proceed as in steps 5, 6 and 7 above.

Manner of Expression of Results

(1) *Method for capillary blood:* Coagulation activity in per cent-thrombotest is calculated by reading off from correlation curve supplied with the reagent, using lower curve.

(2) *Method for venous blood:* Proceed as for capillary blood using upper curve.

(3) *Preparation of correlation curve.* This has been prepared by testing serial dilutions in normal adsorbed plasma of a normal standard reference plasma, obtained by pooling plasma prepared from 10 male and 10 female normal donors. The plasma is first diluted 3/2 with normal saline to give the 100 per cent value for whole citrated blood (the volume of red cells is substituted by saline). The correlation curve for capillary blood is constructed at a 10 per cent lower level. The same curve may be obtained by serial dilution of normal capillary blood using the silicone technic.

Calculations

(1) The ratio between the thrombotest percentage and coagulation time varies somewhat from batch to batch, so correlation curves are not identical. The curve supplied with a particular pack should be used only for tests carried out with ampoules from that pack and then discarded.

(2) Correction for abnormal haematocrit. Correction is normally unnecessary. But, if thrombotest is carried out on patients with severe anaemia or polycythemia, the percentage read from the correlation curve has to be corrected by multiplying it with the correction factor obtained from the curve supplied with the reagent.

Normal range of values. The normal range is 70-130 per cent activity. Lower or higher values may occasionally occur in otherwise normal persons. The average is put at 100 per cent activity.

Therapeutic range in anticoagulant therapy. The optimal level for anticoagulant therapy as controlled by this method is considered to be 15 per cent. By aiming at 15 per cent about 80 per cent of all determinations in stabilized patients can be kept within 10-20 per cent. This range is effective in preventing venous and arterial thrombosis. Bleedings without a local cause do not occur at values above 10 per cent.

Precautions and Sources of Error

(1) *Preparation of reagent.* The pointed top of the ampoules must be removed to let the air in before the ampoule is filed and broken off in the usual place. This must be done to prevent the powder from being blown out by a rapid influx of air. The amount of solvent that is added to the reagent must be measured exactly. Mixing is achieved by short vigorous shaking. The reagent is ready for use within 2-3 minutes of adding the

Steps in the Performance of Test: The test may be carried out with capillary blood or venous blood, but the principle is the same for both methods:

Method for capillary blood

Preparation of reagent:

(1) Remove pointed top of ampoule with forceps, then file and break off in usual place.

(2) Add 11 ml. of lukewarm (20-37°C.) distilled water to large ampoules and 2.2 ml. to small ones. Shake vigorously for a short period of time and reagent is ready for use after 2-3 minutes.

(3) Pipette off 0.25 ml of reagent into small test tubes which are placed in water bath at 37°C. and left there between 3-30 minutes.

Collection of blood.

(4) Collect blood sample from ear lobe or finger pad by making an incision and measuring off 0.05 ml. blood from first drops as fast as possible into pipette

Clotting time determination.

(5) Immediately blow blood sample from pipette into reagent and start stop watch simultaneously.

(6) Mix blood and reagent by flicking once and leave tube in water bath for 30 seconds if normal blood, and for 50 seconds if blood from patients on anticoagulant therapy

(7) At short intervals thereafter, remove tube from water bath and tilt to observe and record moment of coagulation.

Method for venous blood

Preparation of reagent

(1) Proceed as for capillary blood, steps 1, 2 and 3, but use a 3 2 mM solution of calcium chloride as a solvent

Collection of blood

(2) Measure 0.2 ml (or 0 5 ml) of 3 13 per cent sodium citrate (sodium citrate $2H_2O$) solution in a 2 ml (or 5 ml.) syringe and, after venepuncture, fill syringe with blood to a volume of 2 ml (or 5 ml.). Transfer blood immediately to a "non-activating" tube (plastic or siliconized glass) Alternatively, measure the citrate solution into plastic collecting tubes marked for 2 ml. (or 5 ml) volumes, and allow blood to flow directly from needle into tube without using syringe

(3) Measure off 0 05 ml citrated blood into pipette

Clotting time determination

(4) Proceed as in steps 5, 6 and 7 above.

percentage activity. A similar error may occur if the mixture of blood and reagent is made by shaking instead of gentle tilting

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6. Estimation of Prothrombin by the Two-Stage Method

R. H. WAGNER, J. B. GRAHAM, G. D. PENICK and K. M. BRINKHOUS

Object of the Method· The basic object of the two-stage prothrombin assay, devised by Warner, Brinkhous and Smith,[1-3] is the specific determination of prothrombin concentration in any solution, regardless of the presence or absence of other clotting accelerators or inhibitors The assay has been used particularly for the determination of prothrombin in plasma, serum, and plasma fractions Certain other test procedures, as the prothrombin utilization test and a method for measurement of antihemophilic activity, are based on the rate of change in prothrombin during clotting, as measured by the two-stage assay procedure

Principle The assay consists of two stages (1) In the first stage, prothrombin is converted to thrombin as rapidly and completely as possible by incubation with optimal amounts of Ca^{++}, tissue thromboplastin and known accessory plasma cofactors The main plasma cofactors necessary for prothrombin conversion are Ac globulin (factor V), factor VII, and Stuart factor (factor X) (2) In the second stage, the thrombin formed at

solvent. The reagent appears turbid because it is a suspension and not a true solution and, if left standing, must be mixed again before further use.

The small test tube containing the reagent must be left in the water bath (37-39°C.) for at least 3 minutes to reach the correct temperature before the blood sample is added It should not be left in the water bath for more than 30 minutes; otherwise the stability may change.

(2) *Collection of blood:*

Capillary blood. The lobe of the ear or the pad of the finger is washed with ether and a puncture is made which should be sufficiently deep to give a free blood flow. The incision should not be made at the center of the tip of the finger, but at one of the sides, this being less painful. The third or little finger is usually preferred. The *first* drops of blood are used. The blood is preferably collected in a 0.1 ml. pipette with a mark at 0.05 ml. The blood is measured as fast as possible into the pipette which should be held horizontally with its tip in the blood Final adjustment of the exact amount of blood can easily be made by pricking the pipette against the hand (not cotton wool!).

Venous blood

Venous blood should be collected in plastic, lusteroid or siliconized glass tubes in order to prevent glass contact activation of the blood sample. Particularly in cases when the venous blood cannot be tested immediately after collection, it is imperative that non-activating tubes be used for collection and storage.

Citrated blood from patients on anticoagulant therapy may be kept for 48 hours in non-activating tubes before being tested. Citrated blood from untreated persons may be stored for 1 hour only.

Remember that solutions of silicone become less effective on storage, especially on exposure to air.

It is not necessary to use siliconized needles or syringes for the collection of the blood sample, provided that the syringe is emptied immediately.

(1) *Clotting time determination*

The test tubes in which the determinations are performed should be non-siliconized glass tubes The blood is blown into the test tube, holding the top of the pipette just above the surface of the reagent and against the inner wall of the tube. The stop watch is started simultaneously The reagent should not be sucked into the pipette, because the correlation curves provided with each pack have been prepared by the simple blowing-out-technic. This technic has been chosen in spite of the fact that small pipettes are calibrated to contain and not to deliver If blood is allowed to stick to the walls of the tube, mixing will be delayed, causing false low

8 Oxalated plasma. Obtain blood as above, mix immediately w 0.10 M sodium oxalate in a ratio of 1 part sodium oxalate to 9 parts wh blood. Follow rest of procedure as in 7 above.

9. Acacia, 15 per cent solution. Acacia is obtained from S B. Peni New York, N. Y., as Gum Acacia USP White Tears. Dissolve by stirr 60 Gm. acacia in enough hot distilled water to make a 15 per cent so tion. Centrifuge the liquid while warm and strain the supernatant solut through cheesecloth. Adjust the pH to 7.2-7.4, using phenol red as external indicator. Store in the freezer. It was found by Ware and Seege that all lots of acacia tested contained enough Ca^{++} to bring the final c cium concentration within the optimal range when used in the proporti outlined below.

10. Imidazole buffer, pH 7.2. Weigh out 1.72 Gm. C.P. imidaz Edcan Laboratories, South Norwalk, Conn. Dissolve in approximat 90 ml. 0.1 N HCl. Adjust to pH 7.2, dilute to 100 ml. Store in freez

11. Thromboplastins. Three different preparations are used in laboratory. They are equally satisfactory. (a) Crude beef lung throml plastin Five cm. cubes of lung are removed from the periphery of fr beef lungs obtained at the abattoir. Large bronchi and blood vessels removed. The cubes are washed thoroughly under tap water to remove much blood as possible. The lung is ground in a grinder with pores mm. in diameter. The ground lung is weighed and placed in an eq weight of saline This mixture is shaken at intervals and extracted at 5° for 48-72 hours. The mixture is then centrifuged and the supernatant fl is stored in the freezer. The concentrated extract is diluted with norr saline to a point at which maximal thrombin yields are obtained in assay of normal plasma for prothrombin This dilution varies from 5- 20-fold for different preparations. The crude lung thromboplastin pre ration should be assayed for prothrombin to rule out the possibility contamination with this factor (b) Bacto rabbit brain thromboplas (Difco Labs., Detroit, Mich) is prepared according to the manufacture instructions and diluted 5- to 10-fold with normal saline before use (Ultracentrifuged beef lung thromboplastin This preparation is descrit by Ware and Seegers.[4]

12. Fibrinogen All steps in the preparation are carried out at 4° Oxalated dog plasma is adsorbed for 30 minutes with 100 mg $BaSO_4$ p ml Merck U.S.P. or Baker and Adamson Reagent $BaSO_4$ are suitab Centrifuge (about 3000 g) for 30 minutes To the supernatant plasma added one-third volume of cold saturated $(NH_4)_2SO_4$ Let stand for minutes. Centrifuge at 3000 g for 15 minutes Decant supernatant fl and save for factor V preparation (*see below*) Carefully drain tubes co taining the fibrinogen precipitate Dissolve the precipitate by gentle agi

varying incubation times is measured by the speed with which it clots fibrinogen. One unit of thrombin is defined as that amount which will cause the formation of a fibrin clot in 15 0 seconds in 1 ml. of a mixture containing the thrombin, standardized fibrinogen, acacia, imidazole buffer, and Ca^{++}, in an isotonic solution at 28°C. This is the Iowa unit of thrombin. One unit of prothrombin is defined as that amount which, when fully activated, forms one unit of thrombin.

Apparatus: (a) Essential apparatus: (1) Syringes and needles. (2) Centrifuge tubes. (3) Centrifuge. (4) Refrigerator. (5) Balance. (6) Glass tubes; tubes 10 x 75 mm. in size are used in large numbers for the final clotting stage; tubes of larger size are used for storage of reagents (7) 28°C. water bath. (8) Serologic pipettes; a supply of 0.2, 1 and 5 ml. pipettes is needed. (9) Stop watch, preferably operated with a foot pedal. (10) Additional stop watch or clock with a sweep second hand. (11) Cellulose dialyzer tubing (Arthur H. Thomas Co) is used for dialysis at 4°C

(b) Optional Equipment: (1) Freezer, any standard type freezer cabinet capable of maintaining a temperature of −20°C. or lower is useful, the freezer is used for storage of thermolabile reagents. (2) A cold room maintained at a temperature of about 4°C. equipped with a centrifuge and dialysis facilities (3) pH meter.

Reagents· Many different technical preparations are satisfactory. We describe the ones used in our laboratory. Where possible, a list of alternative preparations is given.

1 0 11 M sodium citrate.

2. 0.10 M sodium oxalate

3 0 154 M sodium chloride (normal saline)

4 Citrated saline, 1 volume 0.11 M sodium citrate plus 19 volumes of 0.154 M NaCl.

5. Phenol red, 0 1 per cent solution One-hundred mg. phenol red plus 2.8 ml 0 1 N sodium hydroxide and water to dissolve. Dilute to 100 ml.

6. Saturated $(NH_4)_2SO_4$ solution. One hundredth M potassium oxalate solution is saturated in the cold with $(NH_4)_2SO_4$ and adjusted to pH 7.1-7.2 by the addition of 1 N NH_4OH. pH is checked on a 1-10 dilution of the reagent to reduce salt effects

7. Citrated plasma Obtain blood by venepuncture, using the two-syringe method Avoid air bubbles in collection Mix immediately with 0 11 M sodium citrate in a ratio of 1 part sodium citrate to 8 parts whole blood Centrifuge at about 3000 g for 20-30 minutes Withdraw the supernatant plasma after determining the hematocrit Keep at 4°C. until just before use. If the plasma is not to be tested within 1-2 hours after venepuncture, store immediately in freezer

could be formed from 1 ml. of native plasma, and, by definition, the prothrombin concentration in units per ml.

(1) Thaw 1 ml. of fibrinogen solution and put 0.1 ml. samples in serologic tubes in a 28°C. water bath. Bring other reagents to 28°C.

(2) Dilute 0.1 ml. of the test sample with a mixture which contains 1 part factor V and 39 parts citrated saline. The aim of this step is to reduce the prothrombin concentration of the sample to approximately 5 units per ml. This is the *isometric dilution factor* and it varies with the strength of the sample. For normal human plasma this factor is usually 40 to 50.

(3) Incubation. To 3 ml. of two-stage reaction mixture, add 1 ml. of the diluted sample prepared in (2) above. Mix and note the time. Incubate mixture in a 28°C. bath.

(4) Testing for thrombin. At 30-second intervals, transfer rapidly a 0.4 ml. aliquot of the incubation mixture to a tube containing 0.1 ml. fibrinogen. Simultaneously start the stop watch. Keep the contents of the tube in constant agitation. Clotting is best observed by holding the tube at an angle in front of a light source so that the lower part of the tube only is strongly illuminated. The end point is sharp. If clotting does not occur within 25 seconds, proceed with the next aliquot.

In steps (3) and (4) above the prothrombin test solution is diluted 5 times. This is the *final dilution factor* and it is constant.

(5) If the first dilution tried (step 2) gives no clotting time shorter than 18 seconds when tested for thrombin (step 4), go back to step 2 and prepare a more concentrated solution; if the fastest clotting time is less than 12 seconds, prepare a more dilute solution.

TABLE 1 *Relation of Clotting Time to Thrombin Concentration under Standard Conditions*

Clotting time (seconds)	12.0	12.5	13.0	13.5	14.0	14.5	15.0	15.5	16.0	16.5	17.0	17.5	18.0
Thrombin concentration in units per ml.	1.34	1.26	1.20	1.14	1.09	1.04	1.00	0.96	0.92	0.89	0.86	0.82	0.80

Calculations. The calculations are illustrated by means of an assay of citrated human plasma.

Eight ml. of whole blood were mixed with 1 ml. citrate solution, the cell volume was 3.2 ml. Testing of a 1:50 isometric dilution of the citrated plasma gave the following results.

Incubation time, seconds:	30″	60″	90″	120″	150″
Clotting time, seconds:	15″	14.5″	14.1″	14.7″	15.6″

tion in citrated saline, using one-third the original plasma volume. Dialyze against 3 or 4 changes of citrated saline for 2-3 hours After dialysis, bring pH of fibrinogen to 7.2, using 1 N HCl or NaOH and phenol red as external indicator. Freeze fibrinogen in 1 ml. lots.

13. Factor V[5] *All steps in the preparation are carried out at 4°C.* Measure the volume of the supernatant fluid saved from the fibrinogen preparation. Add one-eighth volume of saturated $(NH_4)_2SO_4$ to bring to one-third saturation, and let stand for 15 minutes in the cold. Centrifuge and discard supernate. Dissolve the precipitate in a volume of citrated saline equal to the starting plasma volume. (See fibrinogen section above.) Dialyze and adjust pH to 7 2; freeze in 5 ml. lots.

14. Beef serum. *Beef blood from the slaughterhouse is allowed to* stand for several hours after clotting. The serum at this time is virtually free of prothrombin. The serum is removed by centrifugation and frozen.

15. Two-stage reaction mixture *This mixture contains thromboplastin,* acacia, calcium, saline and imidazole buffer *It may be prepared with a* number of variations. It is commercially available in lyophilized form, as Bacto-Prothrombin 2-Stage Reagent (Difco Labs., Detroit). This has been manufactured according to the procedure of Ware and Seegers[4] Each ampule is reconstituted by the addition of 10 ml. 0.6 per cent sodium chloride. For each 2.9 ml. of reconstituted mixture add 0.1 ml 1:10 saline dilution of "prothrombin-free" beef serum (not $BaCO_3$-treated beef serum) The following procedure is the one used in our laboratory. (a) Calcium-imidazole-acacia mixture This mixture is made up in large batches and may be stored indefinitely in the freezer. It consists of 2 volumes of 15 per cent acacia solution, 3 volumes of normal saline, and 1 volume of imidazole buffer (b) Reaction mixture. This mixture is made up shortly before use and frozen It consists of 2 volumes of calcium-imidazole-acacia mixture, 0 9 volume of dilute thromboplastin, and 0.1 volume of beef serum diluted 1.10 with normal saline

Steps in the Procedure· The assay consists of the following steps: Preliminary dilution of the test sample with factor V and citrated saline, incubation of the diluted sample with the two-stage reaction mixture; testing of the incubating mixture for thrombin, at intervals, by adding aliquots to fibrinogen and noting the clotting time The shortest clotting time represents the maximum thrombin yield for a given dilution For purposes of calculation, the clotting time must fall in the 12-18 second range. Therefore, the preliminary dilution is varied until the shortest clotting time falls in that range. The thrombin concentration in the final mixture is found from the clotting time and table 1. The product of the thrombin concentration times the total dilution is an expression of the total thrombin that

(2) Thrombin solutions used to remove fibrinogen from plasma may cause partial inactivation of prothrombin The loss is normally less if fresh serum is used as a source of thrombin.

(3) Heparin is not a satisfactory anticoagulant for obtaining plasma samples.

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7. Estimation of the Rate of Prothrombin Utilization

J. B. GRAHAM, R. D. LANGDELL and K. M. BRINKHOUS

Object of the Test This test is designed to furnish an index of the rate of conversion of prothrombin to thrombin during clotting The prothrombin conversion is slow in a number of conditions such as hemophilia and the hemophilioid diseases as well as in thrombocytopenia and some thrombocytopathias

Principle When normal blood clots, prothrombin is transformed into thrombin Because of the inactivation of thrombin by antithrombin, the amount of free thrombin present at any time during clotting is low and is not a reliable index of the efficiency of the clotting reaction By stopping prothrombin conversion of clotting whole blood at timed intervals by the addition of sodium citrate and centrifugation, the amount of prothrombin remaining in serum can be measured by the two-stage prothrombin method At the time the clot is first observed, very little prothrombin has been converted to thrombin Thereafter, prothrombin is converted to thrombin more rapidly, until in a few hours only traces of prothrombin remain

Reagents and Apparatus The same as for the two-stage method for determination of prothrombin (see page 159) In addition to the items

(1) From table 1, 14.1 seconds is found to be equivalent to 1.09 units of thrombin per ml.

(2) $\frac{\text{Volume of citrated plasma}}{\text{Volume of native plasma}} = \frac{9.0 - 3.2}{5.8 - 1.0} = \frac{5.8}{4.8} \approx 1.21$, citrate dilution factor.

(3) Thrombin concentration times citrate dilution factor times isometric dilution factor times final dilution factor $= 1.09 \times 1.21 \times 50 \times 5 = 330$ units prothrombin per ml. plasma.

Values Obtained: Because of the possible day-to-day variations in the potency of reagents used, there may be a day-to-day variation in prothrombin levels in terms of absolute units of prothrombin. For this reason, in work with plasmas, a normal control plasma is assayed at the same time, and all results are expressed in terms of per cent of the control.

The values found for normal human prothrombin usually fall in the range of 300-360 units per ml. In a series of 10 determinations on a sample of human plasma, the concentration was found to be 312 units per ml., with a standard deviation of ± 13 units. In a series of determinations on 10 normal adults, the average level was 336 units per ml. with a standard deviation of ± 32 units.

Alternate Procedure. Defibrinogenation appears to be a desirable but not an essential step unless the unknown sample contains fibrinogen and very little prothrombin When it is omitted from the procedure, a small clot sometimes forms in the incubating mixture If the clot is pipetted, along with the thrombin, into the fibrinogen, it must not be confused with the end point, which is the formation of a large easily visible clot.

Defibrinogenation of plasma to be tested for prothrombin has been previously described[23] It consists of adding dilute commercial topical thrombin or a fresh serum, rich in thrombin and deficient in prothrombin, to the sample to be tested, just before the isometric dilution step (see p. 163) After a few minutes the fibrin clot is rolled out, the plasma antithrombin quickly inactivates the added thrombin. The rest of the procedure is as outlined.

Precautions and Sources of Error (1) When plasmas or sera are being tested, the maximal thrombin yield obtained may be somewhat less than that which would be obtained in an antithrombin-free system, since some of the thrombin formed may be inactivated by antithrombin before it participates in the thrombin-fibrinogen reaction. As a result, when different dilutions of a given sample are tested, the more dilute samples, containing less antithrombin, may give a somewhat higher unitage of prothrombin. For most accurate work, the greatest dilution of sample is found which will still fall within the 18-second range

10-19 per cent, the result is presumably abnormal, but the test should be repeated, paying particular attention to the values at 20 and 40 minutes. If the residual prothrombin is greater than 20 per cent at 60 minutes, prothrombin conversion is impaired.

For human blood, an average of 10 normal subjects gave a value for prothrombin half-life of 36 minutes, with a range of 31-41 minutes. For dog blood, the mean value was 28 minutes.

Precautions and Sources of Error: (a) Care should be taken to avoid air bubbles or undue manipulation in the collection of the blood and its distribution into the clotting tubes. Also, the tubes should be scrupulously clean; care should be taken that they do not become contaminated with silicone, which will change the surface property of the glass. Variable and non-duplicable results are obtained if these precautions are not observed. (b) The clotting tubes after citration should be centrifuged immediately. Prothrombin conversion is not stopped completely until the serum is fully expressed from the clot. (c) If a severe hypoprothrombinemia exists, this test cannot be satisfactorily carried out.

References

[1] Brinkhous, K M: A study of the clotting defect in hemophilia. The delayed formation of thrombin. Am. J. M. Sc. *198* 509, 1939

[2] Buckwalter, J A., Blythe, W B, and Brinkhous, K M Effect of blood platelets on prothrombin utilization of dog and human plasmas Am J Physiol *159* 316, 1949

[3] Langdell, R D, Graham, J B, and Brinkhous, K M Prothrombin utilization during clotting comparison of results with the two-stage and one-stage methods Proc Soc Exper Biol & Med *74* 424, 1950

[4] Graham, J. B., McLendon, W W, and Brinkhous, K M Mild hemophilia an allelic form of the disease Am. J M Sc. *225* 46, 1953

listed for the prothrombin assay, silicone-treated syringes (SC-87 Dri-Film, General Electric) are needed for collection of blood, and 10 x 75 mm. tubes graduated at the 1 ml. mark are required for clotting tubes.

Steps in the Procedure· (a) The two-syringe method is used for collection of the blood. Only blood collected in the second syringe is used in the test. (b) One ml. of blood is distributed into each of 10 clean 10 x 75 mm tubes, graduated at the 1 ml. mark. (c) Add 0.15 ml. 0.11 M sodium citrate solution to each of two tubes at the following five times: 0, 20, 40, 60 and 120 minutes. If the tube has clotted prior to the addition of the citrate solution, gently free the clot from the test tube wall with a wooden applicator stick. Immediately centrifuge at 3000-5000 g for 5-10 minutes (d) Aspirate supernatant citrated serum (or plasma) with a capillary pipette and attached rubber aspirating bulb. Pool the suepernatant serum from each pair of tubes. (e) Freeze and store each sample. Determine the prothrombin content of the samples by the two-stage method. (f) Calculate the prothrombin units per ml of each sample. In the calculation, the citrate dilution factor may be neglected. The values thus obtained are low, but since the citrate dilution factor is constant for the series, the relative values are unaffected The amount of residual prothrombin in each sample is expressed in per cent, using the sample citrated at 0 time as 100 per cent

Alternative Procedures (a) A simplified prothrombin utilization test may be done. The procedure is the same as described above, except that the residual prothrombin is determined only at 60 minutes. (b) Imidazole may be used to buffer the whole blood prior to citration The imidazole buffer is described under the assay of prothrombin by the two-stage method. If the buffer is used, the clotting tubes are graduated at the 1.15 ml. mark. First, 0 15 ml. imidazole buffer is placed in the tubes, then 1 0 ml whole blood, and the tubes are gently mixed Otherwise, the procedure is the same as described above (c) The timed intervals must be adjusted for each species studied For amphibian and avian blood, the period of observation must be increased considerably.

Expression of Results. (a) The results may be expressed merely as the amount of residual prothrombin in per cent remaining at 60 and 120 minutes (b) Alternatively, the results may be expressed as the time required for 50 per cent utilization of prothrombin (prothrombin "half-life"). This time is determined by interpolation after plotting the residual prothrombin in per cent against the elapsed time prior to citration, using rectangular graph paper If less than 50 per cent of the prothrombin is used up during the greatest time interval, the result is expressed merely as a prothrombin half-life of $>$ 120 minutes.

Normal Range of Values. For human blood, the expected residual prothrombin at 60 minutes is $<$ 10 per cent. If the value is in the range of

10-19 per cent, the result is presumably abnormal, but the test should be repeated, paying particular attention to the values at 20 and 40 minutes. If the residual prothrombin is greater than 20 per cent at 60 minutes, prothrombin conversion is impaired.

For human blood, an average of 10 normal subjects gave a value for prothrombin half-life of 36 minutes, with a range of 31-41 minutes For dog blood, the mean value was 28 minutes.

Precautions and Sources of Error: (a) Care should be taken to avoid air bubbles or undue manipulation in the collection of the blood and its distribution into the clotting tubes Also, the tubes should be scrupulously clean; care should be taken that they do not become contaminated with silicone, which will change the surface property of the glass. Variable and non-duplicable results are obtained if these precautions are not observed (b) The clotting tubes after citration should be centrifuged immediately. Prothrombin conversion is not stopped completely until the serum is fully expressed from the clot. (c) If a severe hypoprothrombinemia exists, this test cannot be satisfactorily carried out.

References

[1] Brinkhous, K. M : A study of the clotting defect in hemophilia. The delayed formation of thrombin. Am. J. M. Sc *193* 509, 1939

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8. TAMe Assay for Estimation of Prothrombin Activity (Method of Glueck, Sherry and Troll[1])

Described by R. R. HOLBURN

Object of Test: Assay of the prothrombin content of plasma by measuring the esterase activity of thrombin on the synthetic substrate TAMe.

Principle: Prothrombin is converted to thrombin by calcium and thromboplastin in the presence of low concentrations of TAMe. Thrombin, in the first 5 minutes of the reaction, hydrolyzes the substrate releasing carboxyl groups which are titrated with sodium hydroxide The number of carboxyl groups is directly proportional to the number of thrombin units formed from prothrombin in the plasma[2]

Preparation of Reagents. Collection of blood. 9.8 volumes of blood are collected into 0 2 volumes of 19 per cent sodium citrate. Plasma is separated after centrifugation of the citrated blood at 2400 g for 30 minutes at 4°C. Each 1.8 ml. of plasma is defibrinated by the addition of 0.2 ml. of 100 U /ml thrombin topical in 50 per cent glycerol. The clot is removed by reaming with an applicator stick. The plasma is allowed to stand at room temperature for at least one-half hour for the inactivation of residual thrombin by plasma antithrombin

TAMe (p-tosyl-l-arginine methyl ester HCl) obtained from Mann Research Laboratories, New York, N Y.: 0.4 M and 0 009 M in Tris buffer.

Tris buffer [Tris (hydroxymethyl)aminomethane]. 0.1 M, pH 9 0

Thromboplastin, made up in Tris buffer

$CaCl_2$ 0 02 M in Tris buffer

37 per cent formaldehyde adjusted to pH 9 0 with sodium hydroxide.

Sodium hydroxide 0.005 N

Indicator: 0 01 per cent phenol red

Performance of the Test Blank 0 2 ml 0.4 M TAMe, 0 9 ml. Tris buffer, 0 3 ml thromboplastin, 0 3 ml 0 02 M $CaCl_2$, 0 3 ml. defibrinated plasma. Incubate 30 minutes at 37°C. Add 2.0 ml 37 per cent formaldehyde. *Test* 0 2 ml. 0.009 M TAMe, 0 7 ml Tris buffer, 0.3 ml thromboplastin, 0 3 ml 0.02 M $CaCl_2$, 0 3 ml defibrinated plasma After 5 minutes of incubation at room temperature, add 0 2 ml 0 4 M TAMe. Incubate 30 minutes at 37°C Add 2 0 ml 37 per cent formaldehyde. Centrifuge both tubes for 5 minutes at 2400 g Remove supernatant carefully. Transfer 3 ml aliquots of the clear supernatants to 25 ml. E ,

meyer flasks. Titrate with 0.005 N sodium hydroxide using 0.2 ml. phenol red as indicator.

Calculation: The TAMe unit has been defined as that amount of thrombin which will release 1 μmole of acid from TAMe under the conditions of the test. It is approximately equivalent to the activity of one N.I.H. clotting unit of thrombin. The following equation is used to calculate the number of TAMe units in 1 ml. of plasma: (titration of test in ml. − titration of blank in ml.) x plasma dilution x 1/3 (to decrease to a 10-minute period) x 5 (conversion of standard base to μmoles). Dilution factor includes 30/29 for anticoagulant, 10/9 for defibrination, 10/3 for aliquot of plasma used, 4/3 for aliquot of reaction mixture titrated; a total dilution factor of 5.1.

Normal Range of Values.[1] The mean prothrombin content of the plasma of 25 normal human subjects was 53.3 TAMe units per ml. of plasma with an S.D. of ± 4.5. In six dog plasmas the mean value was 38 TAMe units per ml. of plasma. In four rabbits the mean value was 39 TAMe units per ml. of plasma

Precautions and Sources of Error: The period of thrombin formation must be regulated carefully. There should be maximum thrombin formation present and aliquots withdrawn before antithrombin acts to decrease thrombin activity. Adequate factor V (25 per cent or more by the one-stage prothrombin time) is necessary to ensure rapid and complete thrombin evolution.

The presence of 0.4 M TAMe in the blank is inhibitory for thrombin formation thereby supplying good control for reagent activity.[2,3] Dilute TAMe in the test mixture is preferentially hydrolyzed before antithrombin inactivates the thrombin formed

The period for destruction of thrombin added for defibrination must be observed, because any excess thrombin will increase the amount of substrate hydrolyzed.

Other proteolytic enzymes, such as plasmin, may interfere in the assay by hydrolyzing the synthetic substrate.

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9. A Spectrophotometric Modification of the TAMe Assay for Plasma "Prothrombin"

D. A. HOUGH, L. V. LYONS, J. L. KOPPEL and J. H. OLWIN

Object of the Method. To provide a means for the control of anticoagulant therapy

Principle. Plasma prothrombin is converted to thrombin by an interaction of plasma with tissue thromboplastin in the presence of diluted, adsorbed bovine serum as a source of factor V. Thrombin is measured in terms of its esterase activity on p-tosyl-l-arginine methyl ester (TAMe). The calculation of per cent prothrombin present in plasma is based on the measurement of acid (p-tosyl-l-arginine) production as determined by means of changes in the absorption of phenol red.

Reagents and Apparatus Required

Thrombin: The contents of one vial of bovine thrombin (Parke Davis, Topical), containing approximately 5000 N.I.H. units, is dissolved in 50 ml. of 0.85 per cent NaCl, divided into 1.0 ml. aliquots, frozen and stored at −40°C. It may be kept under these conditions for at least 6 months.

Tris buffer. Sigma 7-9, 0.025 M, pH 8.2, dissolved in distilled, demineralized water (pH adjusted with 2 N HCl)

TAMe: 0 2 M, dissolved in Tris buffer, pH adjusted to 8 2 with 2 N NaOH

$CaCl_2$: M/40, dissolved in distilled, demineralized water

Phenol red. 0 015 per cent dissolved in Tris buffer

Beef serum: 50 ml of 1 9 per cent $CaCl_2$ are added to 500 ml of freshly thawed beef plasma and from the resulting clot serum is expressed through heavy gauze In order to remove any remaining prothrombin as well as factors VII, IX, and X, the serum is adsorbed with powdered $BaCO_3$ (200 mg. per 5 ml) A paste is made of the barium carbonate and a small amount of serum, the remainder of the serum is added and the mixture is stirred for 10 minutes at room temperature Finally, the barium carbonate is removed by centrifugation The freshly prepared $BaCO_3$-adsorbed beef serum is subsequently diluted 75 times with Tris buffer

Beef lung thromboplastin One frozen beef lung is allowed to thaw at room temperature The lung tissue is separated from the bronchi, the surface blood is rinsed off with several washings of water and the lung fragments are ground in a meat grinder Five hundred ml of 0 85 per cent NaCl are added to 1000 Gm of beef lung and the resulting mixture is allowed to incubate with occasional stirring at 5°C for 48 hours. It is then

strained through heavy gauze and the extract obtained is put up in tubes, frozen and stored at −40°C. Prior to use it is thawed and diluted 1:16 with Tris buffer.

Activation mixture: The activation mixture (total volume 5100 ml.) is prepared by combining the following reagents:

9 6 parts 16 times diluted beef lung thromboplastin
8 4 parts 75 times diluted beef serum
9 parts calcium chloride
3 parts phenol red
21 parts Tris buffer

The resulting mixture is distributed into 25 ml. tubes, frozen and stored at −40°C. The preparation is allowed to stabilize for 1 month at that temperature before being used. Storage under these conditions for as long as 6 months has not resulted in any change in activity

Lyophilization of reagents: Both the TAMe solution and the activation mixture have been lyophilized and found to remain stable and uniformly active for a minimum of 18 months when stored at 4°C

Standardized normal human plasma (SNP-Dade Reagents, Inc).

Test tubes (10 x 75 mm.), graduated pipettes (0.1, 1.0 and 2 0 ml), Coleman cuvettes (12 x 75 mm.), clinical centrifuge, water bath, stop watches with foot pedal control, Coleman Jr. spectrophotometer, Parafilm (Grade M, Marathon)

Steps in the performance of the test Three ml of blood are collected by means of the two-syringe technic and 9 parts of blood are added to 1 part of 3.2 per cent sodium citrate The mixture is gently inverted several times to insure proper mixing The samples are centrifuged at room temperature for ten minutes at 1650 g and the plasma is drawn off and stored at 4°C. until ready for use.

A 0 4 ml. volume of each plasma to be assayed is placed into a 10 x 75 mm. test tube and defibrinated by the addition of 0 04 ml of thrombin Incubation is carried out at room temperature and the fibrin clot is rolled out exactly 4 minutes after the addition of thrombin The defibrinated plasma is allowed to stand for an additional 15 minutes at room temperature.

One-tenth ml of the defibrinated plasma is then added to 1 7 ml of activation mixture in a 12 x 75 mm Coleman cuvette The cuvette is placed in a 37°C water bath and 0 2 ml. of TAMe is added to it immediately A piece of parafilm is placed over its open end and the tube is inverted several times to assure adequate mixing The per cent transmittance of the mixture is then measured at 565 mμ using a distilled water blank set to read at 100 per cent Following incubation of the mixture for exactly 30 minutes at 37°C, its transmittance is read again, the two transmittance readings are converted to optical density (O D) values, and

the 0-30 minute change in O.D. is determined. A detailed description of the procedure followed when a number of plasma samples are to be assayed simultaneously, follows:

To five 10 x 75 mm. test tubes, plasma samples are added in this order.
Tube #1—0.4 ml. SNP* (reconstituted plasma)
Tube #2—0.4 ml. patient #1 plasma
Tube #3—0 4 ml. patient #2 plasma
Tube #4—0.4 ml. patient #3 plasma
Tube #5—0.4 ml. patient #4 plasma

For the defibrination of the plasmas, 0 04 ml. of thrombin is added to each tube using a 0.1 ml. calibrated pipette.

1. Thrombin is blown into tube #1, its contents mixed gently by hand, and a stop watch is started.
2. At *2 minutes* on the stop watch, thrombin is blown into tube #2 and its contents mixed.
3. At *4 minutes* on the stop watch, thrombin is blown into tube #3 and its contents mixed. Immediately after, the clot formed in tube #1 is wound out.
4. At *6 minutes* on the stop watch, thrombin is blown into tube #4 and its contents mixed Immediately after, the clot formed in tube #2 is wound out.
5. At *8 minutes* on the stop watch, thrombin is blown into tube #5 and its contents mixed. Immediately after, the clot formed in tube #3 is wound out
6. At *10 minutes* on the stop watch, the clot formed in tube #4 is wound out
7. At *12 minutes* on the stop watch, the clot formed in tube #5 is wound out

Following this, a calibrated 0 1 ml pipette is placed into each of the five defibrinated plasma tubes

Note The above procedure for adding thrombin can be carried out at 1 minute intervals when sufficient familiarity with the assay has been achieved However, the clots should continue to be wound out approximately 4 minutes after the addition of thrombin This makes it possible to run 10 assays at one time

One and seven-tenths ml of activation mixture is then added to each of five 12 x 75 mm Coleman cuvettes These tubes should be pre-warmed for approximately 30 seconds in a 37°C water bath before the addition of defibrinated plasma

8. At exactly 15 minutes on the stop watch, 0 1 ml defibrinated plasma from tube #1 is added to a Coleman cuvette containing 1.7 ml. of

* SNP—Standardized Normal Human Plasma (Dade)

activation mixture. (*Note:* The pipette should be washed out at least once with the activation mixture.) Then 0.2 ml. of TAMe are added immediately. A parafilm square is placed over the open end of the cuvette before the latter is inverted to insure complete mixing The per cent transmittance of the mixture is read against a distilled water blank set at 100 per cent. The reading is recorded and a second stop watch is started. The cuvette is subsequently returned to a 37°C. water bath.

The above procedure is continued at 2 minute intervals until the transmittances of all five mixtures have been determined and recorded Care must be taken to record the time on the second stop watch whenever a mixture's transmittance is being read.

The cuvettes are incubated in a 37°C water bath. Each tube is read a second time *exactly 30 minutes* after the respective initial reading With parafilm squares placed over their open ends, the cuvettes are again inverted gently prior to being read.

Calculation and Expression of Results The transmittance readings are converted to optical density (O.D) values. The 30-minute O D values are subtracted from the corresponding 0-minute values and the resulting O D difference is divided by a "factor" for the particular set of reagents used The "factor" is obtained by dividing the O D difference obtained for a control plasma by the per cent prothrombin concentration of the latter. Thus, the "factor" in the example below is:

$$\frac{\text{O D difference for SNP (control)}}{\text{Per cent prothrombin for SNP (control)}} = \frac{0\,238}{70} = 0\,0034$$

	0-Minute Transmittance	30-Minute Transmittance	O D Difference	Per Cent Prothrombin
SNP (control)	22*	38*	0 658 0 420 0 238	70 0
Patient #1	22*	26*	0 653 0 585 0 068	20 0
Patient #2	24*	41*	0 620 0 379 0 241	70 9

To calculate the per cent prothrombin in the plasma from patient #1

$$\frac{\text{O D difference for plasma \#1}}{\text{"factor"}} = \frac{0\,068}{0\,0034} = 20 \text{ per cent}$$

A similar calculation is made for patients #2, #3, and #4

The SNP control plasma has been found to have a prothrombin concentration of 70 per cent and this concentration has been found to be quite stable from lot to lot. Over a period of 2½ years, approximately 25 different lot numbers were tested and the prothrombin concentration was always found to be betwen 68 and 72 per cent.

The SNP control plasma has to be assayed only once with each new lot of reagents. Once the "factor" for a particular set of reagents has been determined, it can be used from day to day as long as the particular set of reagents is being used.

Normal Range of Values: The normal range of values has been 80 to 100 per cent.

Precautions and Sources of Error: Because of the interference of heparin with various phases of the coagulation mechanism the assay cannot be used on heparinized plasmas Owing to interference with observations of optical density changes, the TAMe assay cannot be used with hemolyzed or extremely fatty plasmas.

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10. Preparation and Purification of Prothrombin

W. H. SEEGERS

The main steps in the method for production of prothrombin consist of precipitation of the prothrombin from plasma at an acid pH followed by adsorption on magnesium hydroxide, elution and then fractionation with ammonium sulfate and final isoelectric precipitation.

Reagents and Materials

Plasma: The blood for this plasma is collected in a special anticoagulant consisting of 1 85 per cent potassium oxalate ($K_2C_2O_4 \cdot 2H_2O$) and 0 5 per cent oxalic acid ($H_2C_2O_4 \cdot 2H_2O$) One part of anticoagulant to nine parts of blood is used. After collection, the plasma is obtained as soon as

possible and either stored in a deep freeze or used at once. If human plasma collected in the usual anticoagulant is used, the plasma is first dialyzed for one hour against cold water, to reduce the anticoagulant concentration.

Oxalated saline: 0.75 Gm $K_2C_2O_4 \cdot 2H_2O$ is mixed with 0.85 Gm. NaCl and dissolved in 100 ml. of water. Larger amounts of this material can be prepared and kept on hand for use, if much prothrombin is to be made.

Magnesium hydroxide cream Slowly, and with constant stirring, 5 liters of concentrated NH_4OH are added to 20 liters of 20 per cent $MgCl_2$. The precipitate is allowed to settle and is washed several times in the water to remove the ammonia. Five hundred Gm. of the centrifuge packed $Mg(OH)_2$ is suspended in 1 liter of H_2O. A commercially prepared paste, "Hydro Magma Parte" (Dow Chemical Company) may be used instead of the preparation described It is used in the proportion of 1 2 with water.

Ammonium Sulfate: A saturated solution having a specific gravity of at least 1:26 is prepared, by saturation, at room temperature.

Method. The procedure described is a two-day procedure for the processing of 6 liters of plasma The amounts can be less but the time required is about the same for smaller quantities. By overlapping, four products can be made in five days. This is done by starting the second product while finishing the first on the second day, etc.

First Day. Two large Pfaudler tanks or large metal containers are partially filled with cold tap water to a volume of about 40 l each The plasma should be diluted about 15 times and when 3 l. of plasma are used this will be the amount of water necessary. Cracked ice is added and the water agitated vertically with a special paddle until a temperature of 0°C is achieved At this point the ice is removed and three liters of plasma that has been strained through saline washed gauze is added to each tank This gives a final plasma-water ratio of 1–15 The water and the plasma is mixed well with the paddle. The pH of the solution is adjusted to 5 1 or 5.2 by the addition of cold 1 per cent acetic acid, thoroughly mixing the acid with the diluted plasma before checking the pH When all the required acetic acid is added the tanks are covered and allowed to stand for three to four hours to allow the proteins to settle to the bottom The supernatant is then removed by suction, being very careful not to disturb the protein layer. When as much of the supernatant has been removed as it is possible to get, the prothrombin, with other materials precipitated, is drawn off from the base of the tank

This precipitate is then centrifuged in the cold at 500 to 600 g This can be done in 100 ml centrifuge tubes in a refrigerated centrifuge or in any other device which will accommodate the volume After spinning, the supernatant is discarded and the precipitate is placed in a Waring blender.

The tubes are washed out with a small amount of oxalated saline and this wash fluid is added to the blender. More oxalated saline is added to a portion of the mixture while in the blender. No more than is necessary to obtain an adequate mixing of the saline and the protein is added. The blender is then used for a period of time long enough to procure complete mixing without foaming and then the suspension is transferred to a beaker in an ice bath while the remainder of the material is treated in like manner. A minimal amount of oxalated saline is used to clean out the blender and this is then added to the beaker.

A mechanical stirrer is now placed in the beaker and glass pH electrodes are dipped into the solution With constant stirring 0.1 N NaOH is added slowly until the pH is raised from an initial level of about 5.5 to 6.4. The alkali must be added slowly to prevent local denaturation, care being taken to avoid suspension of the material in the foam at the surface. All pH determinations require turning off the mechanical stirring motor and being sure that the electrodes are immersed in the liquid.

This neutralized solution is now placed in 100 ml. centrifuge tubes precooled in an ice bath They are spun in the cold for five minutes at 3,000 to 3,500 g. Following this, the supernatant liquid is decanted, through saline washed gauze, into a large beaker. To this is added the magnesium hydroxide cream previously prepared and stored in a refrigerator. Use 225 ml of $Mg(OH)_2$ to 21 ml. of supernatant, or proportionate amounts if the quantities are less Thorough mixing of this suspension is essential, because at this stage the prothrombin is adsorbed on the magnesium hydroxide particles This is centrifuged at 500 g for 20 minutes to bring down the particles The supernatant is discarded and the white precipitate is transferred to a cooled Waring blender with about 350 ml of cold physiological saline that has been used to wash out the centrifuge tubes. This is now mixed thoroughly to wash the $Mg(OH)_2$. This mixture is transferred to the cold 100 ml centrifuge tubes and spun in the cold at about 2,000 g for 5 minutes. The supernatant is discarded, then the precipitate is removed using a glass rod to break it up and then washing out with portions of a total volume of 350 ml. of cold 0 85 per cent NaCl It is then poured into a Waring blender and mixed thoroughly Excessive foaming is to be avoided. The suspension is then replaced in the centrifuge and spun at 2,500 g for 5 minutes, removed with 0 85 per cent NaCl and stirring rod as before, after discarding the supernatant After this final washing, it is blended with about 400 ml of cold 0 85 per cent NaCl. A thorough mixing is important at this stage to place the $Mg(OH)_2$ and adsorbed prothrombin in a fluid suspension. When complete suspension is obtained, the mixture is transferred to a special pressure bottle.

This bottle is a heavy walled 1 liter Seltzer bottle which has been covered with surgical tape as reinforcement to minimize danger of breakage. It is fitted with a special pressure cap which can be tightened to prevent the loss of gas when pressure is built up within. This cap is attached to a tank of CO_2 by pressure tubing

After the solution has been placed in the bottle, the cap is attached and the pressure raised to 40 lb. after preliminary flushing twice with carbon dioxide to remove all air. The bottle, with pressure tube and all is enclosed in a metal protective shell and then placed on a shaker and agitated for 30 minutes After this time, the bottle is removed and placed in an ice bath. The pressure is maintained until the apparatus has cooled, at which time it is reduced slowly to prevent excessive foaming. The mixture is then placed in a metal beaker, covered, and placed in the refrigerator overnight.

Second day· The beaker is removed from the refrigerator and the contents strained through 6 layers of oxalated saline washed gauze into a 500 ml. graduate, by which the volume can be determined. To get all the eluate, gentle manual squeezing of the gauze is most efficient.

A salt and ice bath is prepared to obtain a low temperature of about −10°C. and a 2 l. beaker well wrapped with gauze or towelling as insulation is placed in the bath which has been similarly equipped with protective wrappings. The eluate is poured into the beaker while constant mechanical stirring prevents freezing at the sides of the beaker When the temperature of the solution has reached 0°C., saturated ammonium sulfate is added dropwise so that the temperature does not rise. A volume of ammonium sulfate is added equal to the volume of the eluate in order to obtain a final concentration of 50 per cent saturation When all of this chemical has been added, the beaker is transferred to an ordinary ice bath and then the contents are poured into 100 ml. centrifuge tubes, precolled in an ice bath. The tubes are centrifuged in the cold at 2,500 g for 15 minutes The supernatant is then decanted into a 2-liter metal beaker. The beaker is placed in the salt-ice bath as before and stirring resumed When 0°C has been reached, more ammonium sulfate equal to the original volume of the eluate is added slowly This should give a final concentration of 67 per cent When all the sulfate has been added, the mixture is poured into a precooled glass beaker in an ice bath This is allowed to stand for 20 minutes during which time crystals of magnesium salts are formed which sink to the bottom of the vessel 100 ml. centrifuge tubes are filled with the liquid above the crystals, avoiding them as much as possible The tubes are centrifuged at 5°C. at 2,500 g for 20 minutes The supernatant is discarded

The beaker containing the rest of the mixture not centrifuged this first time, is then emptied of liquid, being very careful to obtain the layer of fluid just above the crystals, for it is here that most of the protein is concentrated and as much of it as possible must be removed. These tubes are then centrifuged as were the first ones. The supernatant is again discarded and the tubes washed out individually with distilled water. This washing should be done carefully by keeping the tubes inverted after pouring off the supernatant. A stream of water is directed at the sides of the tube from below through the mouth of the tube The precipitate must not be touched by the water.

After washing, the tubes are placed in an ice bath. The precipitate in the first tube is broken up with a stirring rod and three to five ml. of distilled water are added. This is transferred to the second tube and the process repeated until all the tubes have been rinsed. The material from the last tube can be removed quite conveniently with a hypodermic syringe fitted with a 15 gauge needle or similar inmplement Next the tubes are rinsed in serial fashion with three to five ml. of water and the final volume collected in the same manner

The precipitate removed is then dialyzed against the de-ionized water to remove the ammonium sulfate. This may be done with the usual laboratory dialyzing apparatus or modifications thereof. We have developed a special apparatus consisting of two disks of DuPont cellophane supported by a cast aluminum frame and the whole rotated in cold deionized water. This is more efficient than the usual Visking casing dialysis bag kept in motion in a water bath In either system, the water should be changed frequently, in order to achieve complete and rapid removal of the ammonium sulfate.

A specific resistance of 1,700 ohms or higher should have been reached in about three hours. If it is 500 ohms or higher the resistance can be raised by the addition of distilled water or de-ionized water until the desired resistance is attained. An equal volume of water will almost double the resistance If the resistance is less than 500 ohms the dialysis will have to be repeated.

When the desired resistance is achieved, the prothrombin solution is put in a 10 ml beaker which has been placed in an ice bath This is the first step of the isoelectric precipitation of prothrombin, making use of the relative insolubility of prothrombin in water solutions at isoelectric point.

Dropwise, 0.25 per cent HCl is added from a micropipet with constant mechanical stirring until a pH of 5 35 or 5 4 is reached The solution is then centrifuged in a 100 ml. centrifuge tube at 2,500 g for 5 minutes to remove the fine cloudy precipitate which contains impurities

The supernatant is placed in a beaker, cooled with an ice bath as before and more acid is added in the same manner with constant stirring until

the pH is 4.6 Precipitation of prothrombin will give the solution a milky appearance. This suspension is transferred to a centrifuge tube and spun at 700 g for three minutes. The supernatant is discarded and the sediment is broken up with a stirring rod and about five ml. of distilled water added slowly. This suspension is then placed in a 50 ml beaker in an ice bath along with approximately five ml. of saline used to rinse the centrifuge tube. With constant mechanical stirring 0 1 N NaOH is added until the pH is raised to 6 5 or 7.5.

The final volume is now transferred to a weighed Erlenmeyer flask by means of a five ml. pipet in order to keep track of the volume. Three ml. of distilled water is used to rinse the beaker, the pH electrodes and the stirrer and then added to flask.

The final volume is recorded and 0 5 ml. is removed for analysis. The remainder can be frozen or freeze dried for storage in a dessicator

Careful work and attention to detail should result in a product having approximately 23,000 to 28,000 units of specific activity per mgm of tyrosine and containing 12,000 to 14,000 units per ml of prothrombin. Variations above and below this arbitrary standard occur due to the use of different lots of plasma. When technical errors are at a minimum there still exists a variation which can only be ascribed to the initial material

Should the analysis reveal a product of poor quality it can be raised by partial adsorption with barium carbonate. This process will raise the specific activity but decrease slightly the total number of units contained, due to the adsorption of prothrombin on the barium carbonate with successive exposures to the adsorbent. A product can be raised from 26,000 or 27,000 to 29,000 or even higher, but further purification is not usual in a product already of 28,000 units or more per mgm tyrosine. To use the barium carbonate to adsorb impurities, about 20 ml of the prothrombin is shaken with 1 gm of barium carbonate The barium carbonate is removed by low speed centrifugation Repeating the adsorption may be tried, but it has been found that a single mixing with barium carbonate seems to be as efficient as many In fact, more than three or four will lead to loss of activity.

Chromatography with Amberlite Amberlite IRC-50(XE-64) of particle size that passes through a 200-mesh screen is used in a column 2 2 cm I D × 80 cm long The resin is equilibrated with 0 1 M phosphate buffer, pH 5.95. About 100 mg of the prothrombin are dissolved in 5 ml of the phosphate buffer first adjusted to pH 5 60 with a little 0 1 N HCl The flow rate is adjusted to 3 to 12 ml./hour, and the work can be done at room temperature The recovery of prothrombin from the column is usually complete. High activity material is obtained if the purity of the starting prothrombin is not appreciably less than 20,000 U /mg tyrosine

Chromatography with DEAE Cellulose: For this work the following buffers are needed:

Buffer A, 0.3 M pH 8
2.194 Gm. $NaH_2PO_4 \cdot H_2O$
40.333 Gm. Na_2HPO_4

Buffer B, 0.05 M pH 7
2 691 Gm. $NaH_2PO_4 \cdot H_2O$
4.331 Gm. Na_2HPO_4

Buffer C
Mix 1 part buffer A
9 parts buffer B
(0.075 M, pH 7.2)

Buffer D
Mix 5 parts buffer A
5 parts buffer B
(0.175 M, pH 7 45)

The anion exchanger was obtained from Brown Company, Berlin, N H The cellulose is conditioned by washing several times with buffer B The nonsedimenting material is removed by decantation The bottom of the column is closed with filter paper The cellulose is then mixed a few minutes in a Waring blender, and poured as a slurry by repeated additions until there is no more settling by gravity Air pressure (10 p s.i.) is then applied to obtain a column 25 cm long and 9 mm in diameter. The column then does not run dry under gravity

About 100,000 units prothrombin are taken up in 4 to 5 ml. of the 0 05 M, pH 7 0 phosphate buffer and applied to the top of the column After about 10 to 15 minutes, the prothrombin solution goes in and can be followed with the phosphate buffer at a rate of about 10 ml /30 minutes. (1 drop every 18-20 seconds) About 40 ml. of buffer are run through (collect in four tubes) Then a change is made to buffer C. To do this the 5 ml of the original buffer above the cellulose are not removed, and 70-100 ml of buffer C are run through with no change in rate (1 drop every 18-20 seconds) Then buffer D is used to elute the prothrombin The switch to this buffer is made in the same way as described for buffer C. The prothrombin usually comes out after 30-40 ml of buffer D have been used, and the material with highest potency is in the fourth or fifth tube The column is used at ice box temperature.

Analysis

Prothrombin: All analyses of prothrombin products are done by modified two-stage procedure as described elsewhere (page 159).

Specific Activity: The units per mg of tyrosine are determined means of the Folin-Ciocalteau reaction using 0 1 to 0 2 ml. of the produ From the value obtained by this procedure and the number of units ml as obtained by the prothrombin analysis, the final number of ur per mgm. of tyrosine can be calculated. Units per mgm of dry weight c be found by determining the weight of the product after freeze drying the tared Erlenmeyer flask.

If a freeze drying method is not available, the prothrombin can dried with acetone and stored in a dessicator. There will be a loss of activ of the final product by acetone drying and an analysis will have to be do on the product after drying to obtain the final activity before stora This is not true of the freeze dried material. Analyses should always done before analytical use after either procedure, because there is a grad loss of potency in all products during storage.

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11. Preparation, Purification and Assay c Thrombin

W. H. SEEGERS

Thrombin of good quality can be obtained by the autocatalytic activ tion of purified prothrombin in 25 per cent sodium citrate solution Activ tion by this means makes it possible to avoid the introduction of calciu ions, partially purified Ac-globulin, and tissue extracts with their multip components

If prothrombin is activated with the use of calcium ions and clotti factors, it is well to select reagents that have high procoagulant potenc

A concentrate of Ac-globulin and the sediment obtained by centrifuging lung extracts at high speed makes a good combination. One can also use a concentrate of autoprothrombin II together with calcium ions and purified platelet factor 3. After the full thrombin activity has developed with the use of clotting factors, the thrombin can be precipitated by chilling and adding an equal volume of cold acetone.

The thrombin, in citrate solution, is precipitated by increasing the concentration of sodium citrate to 40 per cent. This is done by adding crystals of sodium citrate. The precipitate is collected by centrifugation and dissolved in water up to the original volume. Since it is not easy to remove the salt by dialysis, the protein is precipitated by adding an equal volume of acetone which is first cooled to sub-zero temperatures. The thrombin solution is stirred as the acetone is added The precipitate is again dissolved in water and once more precipitated with cold acetone. This protein is dissolved in buffer and is then ready for chromatography on IRC-50 resin or on phosphate cellulose.

Materials

Prothrombin. Prothrombin used as the substrate is prepared in the laboratory by the magnesium hydroxide adsorption method described elsewhere (p. 174). It consists essentially of the precipitation of the prothrombin from dilute acidified plasma, its adsorption on magnesium hydroxide; elution, followed by precipitation with ammonium sulfate and a final isoelectric precipitation from an aqueous solution. The products will vary in purity from one preparation to another. Moreover the use of proportionately large amounts of magnesium hydroxide gives high yields of prothrombin, whereas small quantities of the adsorbent give lower yields and higher purity. For some studies one selects the purest obtainable, for as the proportion of impurities increase in the substrate, the excellence of the thrombin product decreases. It is of interest to note, in connection with the last statement, that prothrombin products which have lost much activity as judged by the usual two-stage method of analysis involving physiologic activation, can often be transformed to thrombin in sodium citrate solution. There are derivatives in the prothrombin which, though not detectable by activation with Ac-globulin, thromboplastin and calcium, are capable of becoming thrombin in the autocatalytic reaction.

Activation with sodium citrate Purified prothrombin is dissolved in 25 per cent sodium citrate solution so that the final concentration of prothrombin is about 1—1 5 per cent. To start the autocatalytic reaction, thrombin may be added so that the ratio of thrombin to prothrombin units is about 4 to 100 respectively The solution is then allowed to stand at room

temperature until no more thrombin forms. There may be activatio about 75 per cent of the prothrombin to thrombin within 6 hours. Then may proceed in a variety of ways to obtain thrombin from this mixt which may have a volume of 25 ml.

Chromatography on Amberlite: To carry out the chromatographic f tionation, the following procedure is used: The IRC-50(XE-64) is eq brated at pH 7.0 with 0 05 M sodium phosphate buffer. To make buffer, mix 2.691 Gm. $NaH_2PO_4 \cdot H_2O$ and 4.331 Gm Na_2HPO_4 in w to a liter. A column 2.2 × 12 cm. is poured. About 200,000 to 250, units of thrombin are dissolved in 2.5 ml. 0 05 M sodium phosphate bu pH 7.0, and applied to the column. At the moment when the thrombin gone into the resin, another ml. of the 0 05 M pH 7.0 buffer is caref placed on top of the resin. When this has gone in the resin, the buffe layered over the column in quantity and pressure is applied to give abo drops per minute. When 5 to 15 ml. has run through, carrying with it protein and autoprothrombin C, the operation is stopped for the purp of switching to a buffer of 0.3 M pH 8.0. To make this buffer, diss 2.195 Gm. $NaH_2PO_4 \cdot H_2O$ and 40.333 Gm. Na_2HPO_4 in water up t liter The first buffer is removed down to the surface of the resin and placed with the second The flow rate is again adjusted for getting 2 dr per minute The thrombin comes off the column in the range of 160 to ml. of effluent. The chromatography is done at room temperature To move salts, the thrombin, as taken from the IRC-50 column, is precipit in the cold by the addition of ammonium sulphate powder to 75 per cen saturation. The sodium phosphate, being soluble under these conditio remained in solution The thrombin is sedimented with a centrifuge, solved in water, and precipitated by adding an equal volume of acet previously cooled in a dry ice-alcohol mixture The precipitate is dissol in water The acetone precipitation is repeated several times to insure removal of all ammonium sulphate

Phosphate cellulose and the separation of thrombin The developm and marketing of cellulose preparation for ion exchange chromatogra made it convenient to use phosphate cellulose (PW) from Califor Corporation for Biochemical Research, Los Angeles This is an anio exchanger.

The chromatography with PW cellulose is carried through quite eas The reagents needed are as follows

Buffer A, 0 01 M, pH 7.0
0 538 Gm $NaH_2PO_4 \cdot H_2O$
0.866 Gm Na_2HPO_4

Buffer B, 0.05 M, pH 7.0
2.691 Gm. $NaH_2PO_4 \cdot H_2O$
4.331 Gm. Na_2HPO_4

Buffer C, 0.15 M, pH 8.0
1.097 Gm. $NaH_2PO_4 \cdot H_2O$
20 166 Gm. Na_2HPO_4

The particular cellulose PW we used contained 1 meq. of phosphorus per Gm., and was equilibrated at pH 7.0 with buffer A. A column 2 × 13 cm. was poured and packed under gravity. The column was not allowed to run dry. Then, about 150,000 units of bovine thrombin, that already had at least a specific activity of 12,000 units per mg. of tyrosine, was layered over the column. The thrombin volume was usually not more than 3 ml The thrombin adsorbs. This was followed with buffer A (one drop every 7 to 10 seconds) until 80 to 100 ml. of buffer passed. This brought out impurities and very little thrombin. Then buffer B was used to wash away more non-thrombin protein About 60 ml. were used

Finally buffer C was used to chromatograph the thrombin. After about 60 to 80 ml were collected the thrombin appeared rather sharply in a few tubes (10 ml. each) and had a specific activity in the range of 47,000 units per mg. tyrosine. The buffer C was run at a rate of about one drop every 8 to 10 seconds. This is a rapid procedure and is performed at room temperature The thrombin is then freed of salts as described above after the Amberlite chromatography

Assay The number of units of thrombin is determined by a standard analysis in which the thrombin is diluted until it gives a 15-second clot with a standardized soution of fibrinogen. The titration mixture needed for the analysis follows.

Acacia (15 per cent solution)	2 parts
Imidazole buffer (pH 7 2-7 4)	1 part
$CaCl_2$ (0 70 per cent)	2 parts
NaCl (0 85 per cent)	4 parts

This titration mixture gives the same results as the one used in the prothrombin analysis by the two-stage method. For convenience, a standard solution of the prothrombin incubation mixture is kept on hand and when a thrombin analysis is to be done, this is diluted ½ with 0 85 per cent NaCl. Thus there would be 5 ml of saline added to 10 ml of the prothrombin incubation mixture to prepare the thrombin incubation mixture.

A standard 1 per cent solution of fibrinogen is then thawed after removing from the deep freeze and is pipetted into serologic tubes for the test *One-tenth ml.* is used in each tube

For a thrombin analysis the dilutions of the thrombin are made silicone- or paraffin-lined glassware to prevent the adsorption of thrombin glass. When concentrated solutions of thrombin are handled, this adsor tion will involve only relatively few molecules and the final concentratio will not be affected. In dilute solution, however, the adsorption is eas detected because the thrombin involved by adsorption is an appreciab percentage of the total thrombin available. Throughout the work describ below, silicone- or paraffin-lined glassware is used whenever dilute solutio of thrombin are manipulated. The only exception to this rule is with th final clotting mixtures, in which case standard practice ignores the matter adsorption.

In the test, 0 1 ml of the 1 per cent fibrinogen solution is mixed wi 0 3 ml of the titration mixture. Then 0.1 ml of the diluted thrombin blown into the fibrinogen solution from a 0.1 ml serologic pipet Th end point is determined with the aid of a stop watch started at the insta of mixing the diluted thrombin with the fibrinogen and incubation mixtur The endpoint is the same as that for the other clotting tests, the fir appearance of granularity in the clear solution as it is held to the ligh However, in a thrombin analysis, in the absence of thromboplastin, th endpoint is more abrupt in its appearance and is not presaged by increa ing turbidity, as it is when the thromboplastin is used This granularity replaced by a solid clot in a few seconds if this is the true endpoint Shou the granules persist and no clot form this is a false endpoint and the te should be repeated. No incubation is necessary as in the other tests fo prothrombin or Ac-globulin, because the thrombin is already formed an ready to act directly on the fibrinogen

Should the endpoint vary from 15 seconds, the following correctio table, the same as that used for prothrombin analysis, should be used provide the essential correction figure:

Time	Corr factor	Time	Corr factor	Time	Corr factor	Time	Corr facto
11.0	1.50	14.0	1.10	17.0	0.85	20.0	0.7[illegible]
2	1.47	2	1.07	2	0.84	2	0.7[illegible]
4	1.44	4	1.05	4	0.83	4	0.7[illegible]
6	1.41	6	1.03	6	0.82	6	0.7[illegible]
8	1.38	8	1.02	8	0.81	8	0.7[illegible]
12.0	1.34	15.0	1.00	18.0	0.80	21.0	0.7[illegible]
2	1.31	2	0.97	2	0.79	2	0.6[illegible]
4	1.28	4	0.96	4	0.77	4	0.6[illegible]
6	1.25	6	0.95	6	0.76	6	0.6[illegible]
8	1.23	8	0.94	8	0.76	8	0.6[illegible]
13.0	1.20	16.0	0.92	19.0	0.75	22.0	0.6[illegible]
2	1.17	2	0.91	2	0.74	2	0.6[illegible]
4	1.16	4	0.89	4	0.73	4	0.6[illegible]
6	1.13	6	0.88	6	0.73	6	0.6[illegible]
8	1.12	8	0.86	8	0.72	8	0.6[illegible]

Time	Corr. factor	Time	Corr. factor	Time	Corr. factor	Time	Corr factor
23 0	0 65	24.0	0 64	25 0	0 60	30 0	0 40
2	0 64	2	0 63	26 0	0 49	31 0	0 38
4	0 64	4	0 63	27 0	0.47	33 0	0 34
6	0.64	6	0 63	28 0	0 44	35 0	0 31
8	0 64	.8	0 61	29 0	0 43	37 0	0 28

In practice, if the endpoint varies more than the range of 13 to 17 seconds, the test is repeated with another dilution, for experience has shown that the table is not reliable for accurate work outside of this range, presumably because of personal variations in technic, etc., from one technician to another Three determinations are done for each specimen, and they should not vary by more than 0.5 second at most. These tests should be run after the dilution has been allowed to stand at least two minutes, or they will vary. For some reason, endpoints taken during the first minute will be too fast and a higher value of thrombin will be obtained After this initial equilibration time, the thrombin is stable and will remain so for a matter of several minutes. If the dilution tested is not correct and the endpoint not acceptable, then a new dilution will have to be made This can be done using the correction table as a rough guide.

The test is run at room temperatures, if they are not extreme; otherwise at 28°C. using a water bath.

A known control thrombin standard, in 50 per cent glycerol solution, should be used to check all determinations, as reagents may vary from day to day This standard thrombin solution can be stored in the cold and used many times A standard can also be obtained from the National Institute of Health of the United States Public Health Service.

Calculation Calculation of the result is based on the dilutions performed·

Initial dilution × 5 × correction factor = thrombin units/ml.

Multiplication by 5 is necessary because of the dilution of the sample by the titration mixture and the fibrinogen The correction factor is obtained from the table as given.

Precautions When performing the test, it is of the utmost importance to be careful with all the pipets when doing the dilutions and mixing the materials. The pipet tips should be wiped off each time and there should be a crisp clearing of the bore every time a measurement is made. All pipets are "blown out" when measurements are made. Care must also be taken so no droplets of the solutions adhere to the sides of test tubes and do not actually become mixed with the main solution.

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12. Thrombin Generation Test

F. C. MONKHOUSE

Object of Test To follow by a two-stage method the generation of thrombin in recalcified plasma. It has been shown[1] to be a useful alternative to the thromboplastin generation test.

Principle. When calcium is added to citrated plasma, the coagulation reactions are set in motion If, at intervals after addition of calcium, samples of the plasma are added to fibrinogen, the concentration of thrombin at any particular moment can be calculated. It will be observed that thrombin continues to be formed after the plasma has clotted. *Thrombin generation* curves can be constructed which will indicate the rate of thrombin generated and subsequent decay The concentration of thrombin at the peak of generation is a measure of the potential thrombin available. The length of time required for thrombin concentration to reach a peak indicates the rate of thromboplastin formation and/or activity The length of time thrombin activity continues in the incubation mixture is a reflection of thromboplastin formation and antithrombin activity

Materials Collection of blood Blood should be collected in a 0 1 volume of 3 8 per cent sodium citrate solution, preferably using the silicone technic The blood should be centrifuged lightly (1000 rpm for 4 minutes) If high speed centrifugation is used the method can then be adapted for measurement of platelet activity. The plasma must be used when fresh Incubation of plasma should be carried out in clean dry glass tubes, 12 × 100 mm being an appropriate size Clotting times should be measured in clean dry test tubes 10 × 75 mm

Fibrinogen. Any good quality fibrinogen can be used Contamination with *trace* amounts of plasminogen does not effect the results.

Procedure In each of 10 75 × 100 mm tubes, place 0 2 ml of fibrinogen solution Into a tube 12 × 100 mm., pipette 0 8 ml. of the plasma

to be tested and 0.1 ml. of saline. Allow the plasma to come to bath temperature, then add 0.1 ml. of 0.2 M $CaCl_2$. At 30 seconds and 1 minute intervals thereafter, add 0.1 ml. aliquots of the recalcified plasma to the fibrinogen and note the clotting times. By reference to a standard graph prepared previously, the clotting times are converted to thrombin units. If the reaction is to be followed for a longer period of time, a larger amount of plasma should be taken and other volumes should be altered proportionately. The reaction mixture itself will clot at a time corresponding to the calcium clotting time of the plasma. The fluid can be expressed from the clot by the use of a glass rod.

Normal values will vary considerably depending on degree of centrifugation, dilution of plasma, and amount of contaminating thromboplastin. Thus these should be carefully controlled. In figure 1 are illustrated the variations that can occur when different rates of centrifugation are used and when tissue thromboplastin is added. Care in blood sampling, as in most cases, is obviously very important.

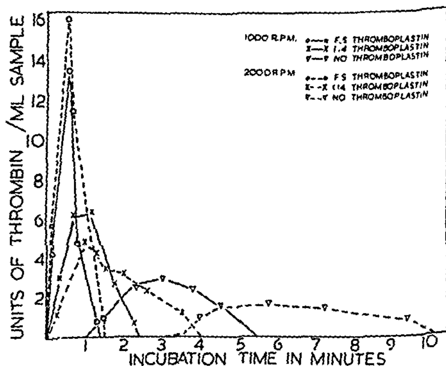


FIG 1.—Effect of rate of centrifugation and addition of thromboplastin on thrombin generation of recalcified citrated dog plasma

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13. The Measurement of Thrombin Activity in Plasma*

G. F. GRANNIS, L. A. KAZAL and L. M. TOCANTINS

Object of Test: To determine the time course of thrombin activity in recalcified plasma.

Principle Aliquots of diluted plasma, taken at various times after recalcification, are added to tubes containing relatively large volumes of fibrinogen solution in sodium citrate buffer. Thrombin generation in the plasma-fibrinogen mixture is arrested by reduction in the amount of available calcium ions, due to the presence of excess citrate, and the rate of thrombin disappearance is greatly decreased by the dilution of plasma with the fibrinogen solution. The plasma-fibrinogen mixture is incubated for a specified time, during which thrombin acts upon the fibrinogen substrate, and the amount of fibrinogen clotted is then determined spectrophotometrically. The amount of fibrinogen clotted is relatable to the amount of thrombin activity added to the fibrinogen solution The thrombin activity thus determined is graphed against time after recalcification to provide a thrombin activity curve (TAC) The latter is useful in evaluating various phases of the coagulation process

Apparatus (a) Plastic test tubes, 12 × 75 mm, (b) glass test tubes, to contain at least 10 ml, (c) about 20 glass rods, prepared in 16 cm. lengths from O D 4 mm glass rod or tubing, (d) stop watch; (e) Beckman DU ultraviolet spectrophotometer, (f) a constant temperature water bath.

Reagents (a) 0 014 M sodium citrate, (b) 0 10 M calcium chloride; (c) exactly 0.25 per cent fibrinogen in 0.014 M sodium citrate † The

* Supported in part by Grant No H3544 from the National Heart Institute, U S P H S, N I H, Bethesda, Md

† Two-tenths ml of 0 25 per cent fibrinogen diluted to 3 0 ml, with alkaline urea solution has an absorbance of 0 275 at 282 mμ when measured in a 1 cm. cell against a blank solution of 0 2 ml 0 014 M sodium citrate diluted to 3 0 ml with rea solution

fibrinogen was prepared from Cohn fraction I of pooled human plasma and purified by precipitation with glycine according to a procedure described for plasma [1] (d) Forty per cent urea in 0.2 N sodium hydroxide, (e) 0 15 M sodium chloride, (f) bovine thrombin (Parke-Davis); 100 units/ml in 0.014 M sodium citrate.

Steps in Performance of Test

1. One and one-half ml. of standard fibrinogen solution is added to each of 20 plastic tubes, and 5 ml. of alkaline urea solution is added to each of 20 glass test tubes. The tubes of fibrinogen solution are placed in a 25°C. water bath.

2. One ml. of platelet-poor citrate plasma is added to 3 0 ml. of 0 014 M sodium citrate in a plastic tube, and 1 ml. of 0.1 M calcium chloride is added to another tube The tubes are placed in the 25°C. water bath.

3. The diluted plasma is poured into the calcium chloride solution with mixing and the stop watch is started.

4. At appropriate time intervals (see table I), samples are removed from the reaction mixture and added to the small plastic tubes of fibrinogen solution The size of sample to be taken depends upon the reactivity of the plasma at the time of sampling. One-tenth ml. is a convenient volume for normal platelet-poor plasma. The plasma-fibrinogen solution is mixed thoroughly and incubated exactly 14 minutes at 25°C.

5. At the end of the latter incubation period, a glass rod is inserted into the fibrin clot and the clot is wrapped onto it Fluid is expressed from the clot by rolling the rod gently against the wall of the tube The rod with adhering fibrin film is then immersed briefly in a tube of 0.15 M sodium chloride to remove fibrinogen and is deposited in a test tube containing 5 ml of alkaline urea solution

6 The fibrin is dissolved with occasional stirring over a period of about 1 hour When solution is complete, the rod is rinsed with 2 ml of 0.014 M sodium citrate, and set aside

7. The absorbance of each fibrin solution is measured in 1 cm. cells in the spectrophotometer at 282 mμ, using a mixture of 5 ml of alkaline urea reagent and 2 ml. of 0 014 M sodium citrate as the blank Since the absorbance is stable for hours, the measurement may be made at any convenient time.

The Preparation of Standard Thrombin Calibration Curves

A thrombin solution of approximately 100 U /ml. in 0.014 M citrate is prepared and assayed by conventional clotting time technic [2] Aliquots of the standardized solution are diluted further in plastic tubes with 0 014 M citrate to furnish solutions having 0 05 to 20 T U /ml. One-tenth ml. aliquots of the dilute thrombin solutions are mixed with 1 5 ml of standard

fibrinogen solution in plastic tubes and incubated in a 25°C water bath. After exactly 14 minutes, the fibrin clots are removed and assayed in the manner previously described The results are graphed as in figure 1 to provide a standard curve relating the absorbance of the fibrinogen clotted to thrombin activity of the aliquot assayed As the curve is reproducible, it can be converted to tabular form Thus, the amount of thrombin activity in an unknown assay can be read directly from the prepared table

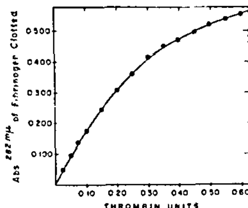


FIG 1—Standard curve relating the amount of fibrinogen clotted to thrombin units See text for details.

Because the thrombin activity of recalcified plasma varies greatly with time, it is convenient to assay aliquots ranging from 0 03 to 0 20 ml. Although the resulting variation in volume of the fibrinogen incubation mixtures is small, it does cause a significant change in the amount of fibrinogen clotted. Consequently, a standard curve should be prepared for each aliquot volume of plasma incubation mixture assayed

After reproducible standard curves have been prepared, thrombin unitage can be defined in terms of the amount of fibrinogen clotted under the standard conditions, and the standardization procedures need not be repeated In this laboratory, 0.5 units of thrombin in a 0 1 ml aliquot causes the clotting of 2 20 mg of fibrinogen under the stated conditions of the determination. The amount of fibrin is calculated using a specific absorbancy, $E_{1cm}^{1\%}$, of 16 5 at 282 mμ, as follows.

$$\frac{C_x}{E_x^{282}} = \frac{C_s}{E_s^{282}}$$

where C_x and E_x^{282} refer to the unknown concentration (mg /ml) and absorbance, respectively, and C_s and E_s^{282} refer, respectively, to the con-

centration (mg./ml.) and absorbance of a 1 per cent fibrinogen solution Since the volume of the solution assayed is 7 ml, the total fibrinogen clotted in the sample is $7C_x$, or:

$$\text{Total fibrinogen clotted} = \frac{E_x^{282} \times 10 \times 7}{16.5}$$

Calculation of Results

Results are expressed in terms of thrombin units per ml. of plasma The amount of thrombin activity in an aliquot can be read directly from the appropriate standard curve, or from the prepared table, and the activity per ml of plasma is calculated by applying an appropriate dilution factor. For example, in the procedure described above, 1 ml. of plasma is diluted with 3 ml. of 0 014 M citrate and 1 ml. of 0.1 m $CaCl_2$. If 0 1 ml aliquots were assayed and if the absorbancy of one such aliquot were 0.500, the thrombin activity read from figure 1 would be 0 46 units. Since the aliquot assayed was one-fiftieth of the volume of the reaction mixture, the latter contained 50 × 0.46, or 23 units of thrombin. Since 1 ml. of plasma had been added to the incubation mixture, the plasma furnished 23 thrombin units per ml at the time the aliquot was taken

The results are graphed as in figure 2 In this illustrative group of experiments, the time course of thrombin generation and disappearance was

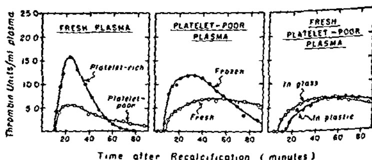


FIG 2.—Typical thrombin activity curves

compared in (a) fresh platelet-rich and platelet-poor plasma, (b) fresh or frozen platelet-poor plasma, and (c) fresh platelet-poor plasma incubated in glass or plastic tubes. These curves demonstrate the general quality of results obtained and indicate that the method is satisfactory for studying the time course of thrombin activity in plasma under various conditions

Precautions and Source of Errors

In addition to the usual precautions in obtaining blood and preparing samples for assay, the following can be listed.

1 In the system described, plasma is diluted with 3 volumes of sodium citrate solution Since approximately 75 per cent of the Ca^{++} requirement

is due to the citrate diluent, minor variations among plasmas will not significantly affect the Ca^{++} optimum of the diluted system. However, when less dilute systems are investigated, the possibility of an altered Ca^{++} optimum should be considered.

2. The timing of sampling and of removing the clotted fibrinogen is critical and a schedule for these operations should be planned before the experiment is begun A schedule which has been found convenient and which permits the simultaneous comparison of two plasmas is given in table 1.

3 Incubation of fibrinogen and plasma aliquot is preferably done in plastic or siliconized glass tubes. In ordinary glass tubes the range of thrombin activity which can be satisfactorily related to clotted protein is decreased, and the reproducibility of duplicate determinations is impaired

4. When whole plasma is assayed, as in the experiments described, the fibrinogen in the recalcified plasma incubation mixture clots shortly after the generation of thrombin is initiated. As representative samples cannot be obtained in the presence of the clot, the clot is either removed as formed by collecting onto a glass rod or the plasma is defibrinogenated before recalcification.

TABLE 1· *Time Schedule of Operations for Simultaneous Determination of Two Plasma Thrombin Activity Curves*

Add Aliquot to Fibrinogen Solution		Remove Fibrin Clot	
Plasma I	Plasma II (minutes after recalcification)	Plasma I	Plasma II
2	3	16	17
5	6	19	20
8	9	22	23
11	12	25	26
14	15	28	29
17	18	31	32
21	24	35	38
27	30	41	44
33	36	47	50
39	42	53	56
45	48	59	62
51	54	65	68
57	60	71	74
63	66	77	80
69	72	83	86
75	78	89	92
87	90	101	104

REFERENCES

[1] Kazal, L A, Grannis, G F., and Tocantins, L M Preparation and purification of fibrinogen Chap IX, p 232
[2] Seegers, W. H, and Johnson, J. F Preparation, purification and assay of thrombin Chap VII, p 181.

CHAPTER VIII

ACCESSORY PLASMA OR SERUM COAGULANT FACTORS

1. Estimation of Accelerator Globulin (One-Stage Method of Lewis and Ware)

Adapted by R. T. CARROLL*

Principle—Formation of thrombin in a system of controlled prothrombin, thromboplastin and fibrinogen content is directly proportional to the amount of Ac-globulin present.

Apparatus and Reagents

(*a*) *Substrate* One unit of outdated dried plasma (Lilly) is reconstituted, using about two-thirds of the saline solution provided, and allowed to stand until the Ac-G deteriorates (about six days) Since deterioration takes place much more rapidly in oxalate than citrate, the reconstituted plasma is divided into lots of 25 ml. and dialyzed at room temperature against 0.02 M potassium oxalate in saline for a period of two days. The contents of the dialysis bags are then pooled, placed in the refrigerator at 4°C., and checked daily for prothrombin and Ac-G. If the prothrombin drops below 60 per cent of normal, the material is discarded. To follow the drop in Ac-G activity, a small aliquot of the plasma (5–10 ml) is adjusted to pH 7.4 with 0 01 N hydrochloric acid, and then tested by using as a substrate in the system described below. The material is considered ready for use when a clotting time of 90–120 seconds is obtained when 0.85 per cent NaCl is substituted for test plasma in the system The substrate is then carefully adjusted to pH 7 4 with HCl, and dialyzed in the cold against 0 02 M sodium citrate in physiologic saline, since the presence of oxalate tends to give difficulty with the end-point at slow clotting times. Portions of about 5 ml. placed in tightly stoppered tubes and stored at −10°C. generally prove satisfactory for a period of several months

* From Proceedings of the Society of Experimental Biology and Medicine 84: 640, 1953.

(*b*) *Thromboplastin-calcium solution:* Stock thromboplastin. 20 ml. of 0.85 per cent NaCl containing 0.4 ml. of 0.1 M sodium oxalate are warmed to 45°C. To the warm saline-oxalate solution is added 1.2 acetone-dried human brain powder (prepared from the cerebrum after removing blood vessels). The mixture is inverted and incubated for 30 minutes at 45°C., stirring gently once during the incubation period. Avoid excess agitation. Remove the larger particles by light centrifugation for a few minutes. The opalescent supernatant is relatively stable for a month or more when stored at −10°C. Avoid thawing and refreezing which often results in a drop of thromboplastic activity. For use in the test, one part of the stock thromboplastin is diluted with 4 parts of 0.85 per cent NaCl. The diluted thromboplastin is then mixed with an equal volume of 0.05 M calcium chloride and allowed to stand at 37°C for about 30 minutes before use. The best results are obtained when the clotting times with normal plasma fall between 40 and 50 seconds. It may be necessary to alter the saline dilution of the stock thromboplastin in order to maintain the clotting times in the optimal range.

(*c*) *Standards:* Either untreated beef plasma or prothrombin-free beef plasma provides a stable source of Ac-G for use as a standard. Since the Ac-G level of beef plasmas may vary, standardization should be carried out by comparison with 5–10 normal human plasma samples in the test system described below. When kept at −10°C, the undiluted beef plasma is stable for months. It should be divided into small portions to avoid repeated freezings and thawings. Unless there is some reason to suspect deterioration, one standardization against normal human plasma is usually sufficient for a given lot of beef plasma.

(*d*) *Preparation of plasma sample to be tested.* Blood is collected by a clean venepuncture. Nine volumes of blood are mixed immediately with one volume of 3.2 per cent sodium citrate (0.109 M). If much difficulty is experienced with the venepuncture, or if there is the slightest evidence of incipient clotting, the sample should be discarded since the formation of even small amounts of serum Ac-G will tend to give erroneous results. The plasma is separated by centrifugation as soon as possible. If the assay cannot be done immediately, the plasma should be stored in the refrigerator to avoid deterioration of the Ac-G. At 4°C, Ac-G is usually stable in citrated plasma for about six hours. For the assay, one volume of plasma is diluted with nineteen volumes of 0.85 per cent NaCl.

If materials other than plasma are to be assayed, suitable dilutions in 0.85 per cent NaCl are prepared so that the clotting times in the assay will fall within the range covered by the standard curve. Interference by barium, citrate, calcium or other contaminating ions should be avoided by preliminary dialysis of the sample against 0.02 M sodium citrate. The

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* From Proceedings of the Society of Experimental Biology and Medicine 84 640, 1953.

2. Estimation of Ac-Globulin Activity by the Two-Stage Method

J. F. JOHNSON and W. H. SEEGERS

Definition: Ac-globulin probably exists in the circulating blood as an inactive protein, and in this state is called *plasma Ac-globulin.* With the addition of thrombin or during the clotting of blood this inert form is changed to the active form, *serum Ac-globulin.* This protein then participates with thromboplastin and other factors in the acceleration of the conversion of prothrombin to thrombin. Further action of the thrombin on serum Ac-globulin probably causes its inactivation and disappearance from the circulating plasma in very few minutes.

$$\text{Plasma Ac-globulin} \xrightarrow{\text{Thrombin}} \text{Serum Ac-globulin}$$

$$\text{Serum Ac-globulin} \xrightarrow{\text{Thrombin}} \text{Inactive Ac-globulin}$$

These observations apply to human, chicken, dog, rat, turtle and guinea pig blood. In cow, rabbit and cat blood Ac-globulin remains in the serum and is detectable as serum Ac-globulin for long periods of time However, if large amounts of thrombin are added to these serums, inactivation of the Ac-globulin also results.

Principle of Method: In order to keep the substrate levels constant and known, an initial prothrombin determination must be done before the plasma level of Ac-globulin can be found. This is done by the two-stage method of Warner, Brinkhous and Smith (see page 159) An additional modification of the method is used for each specimen as well as the one described by the authors. The modification consists of the addition of a diluted beef serum to the diluting physiological saline This serum contains about 90 units of serum Ac-globulin per milliliter and has been diluted initially 75 times to supply an adequate amount of the accelerator for the reaction. By this addition any deficiency of Ac-globulin in the plasma will be corrected and the full activation of all the available prothrombin will be accomplished

Any major difference in the two analyses, that is, between the modified and the unmodified methods for prothrombin determination, indicates that there is a deficiency of Ac-globulin This difference, called the differential, is large when there is a lack of Ac-globulin and small when there is sufficient present There is always a slight differential due to the loss of Ac-globulin during the initial defibrination of the plasma with thrombin when the

dialyzed sample should then be diluted 1:20 with saline solution before introducing it into the assay system.

Steps in the Procedure: The test is carried out in a water bath or a constant temperature block kept at 37°C. The substrate and thromboplastin-calcium mixture are warmed to 37°C. before beginning a series of assays, and may be kept at this temperature for several hours for convenience in running a large series of tests. Most consistent results are obtained if the diluted plasma sample is also warmed to 37°C. before adding it to the reaction mixture. In a clean 10 × 75 mm. glass tube, place 0 1 ml substrate; add 0.1 ml. plasma (diluted 1:20 with 0.85 per cent NaCl); blow in 0.1 ml. thromboplastin-calcium mixture, mix, and note the time for clot formation after addition of this reagent. The Ac-G content of the sample is determined by reference to a standard curve.

Calculation: A standard curve is prepared as follows: (a) Determine the clotting times of 5–10 fresh normal human plasma samples (diluted 1:20 with 0 85 per cent NaCl) in the assay system described above. The average of these values represents the clotting time of the 100 per cent standard (b) Make a 1·100 dilution in saline of the same normal plasma samples, and determine the clotting times. Average the values to determine the clotting time of the 20 per cent standard. (c) Determine the dilutions of the stock beef plasma which will give the same clotting times. This beef plasma may then be used as a reference standard (d) Plot the results on logarithmic paper, with per cent standard as the horizontal axis and clotting time in seconds as the vertical axis Since the values between 10 and 100 per cent fall on a straight line when plotted on logarithmic paper, the use of only two points to construct the curve is adequate For Ac-G values which lie appreciably above the 100 per cent level, a greater dilution of the test material should be employed in order to bring the clotting times within the range covered by the standard curve

The results are expressed in terms of per cent of the normal.

Sources of Error· (1) Type of anticoagulant used The stability of human plasma Ac-G is markedly affected by the type of anticoagulant used. Stability is greatest in citrate, while appreciable deterioration takes place in a matter of hours in oxalate The presence of heparin and versene also hasten deterioration (2) Failure to allow a reasonably constant interval of incubation in the constant temperature bath or block before picking up the tube to observe the end-point may give rise to erratic results. (3) When beef plasma is used as the standard, the clotting time of the 20 per cent standard should be determined within a few minutes, since an appreciable drop in activity may occur. (4) Freezing and thawing the thromboplastin solution repeatedly. (5) Improper collection of plasma as described

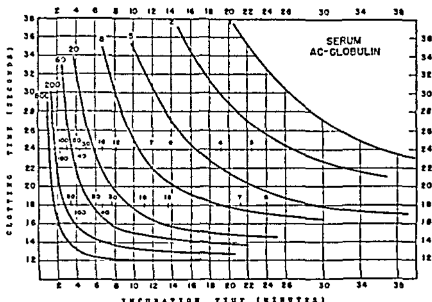


Fig 1

tion is similar to that of a solution of salts seeded with a crystal in which the reaction proceeds at a constantly increasing rate

Bovine plasma is not quite as sensitive to the change in calcium as is human plasma, but there will be definite increase in the amount of Ac-globulin detected, with the reduction of the calcium from the amount present in the incubation mixture used in the two-stage prothrombin test It is possible to use the same incubation mixture for both determinations, if the above cautions are kept in mind Also to be noted is the fact that this lower concentration of calcium is not the optimum for the complete conversion of prothrombin, as in the two-stage prothrombin analysis, and compensation for this should be made when the dilutions of the plasma are made in preparation for an Ac-globulin analysis The proper evaluation of the plasma can be obtained by performing the two-stage prothrombin analysis using the Ac-globulin incubation mixture Values will be lower but they are the ones that will be available in the test for the accelerator

Preparation of plasma and serum for two-stage analysis of Ac-globulin

Collection of the blood When collecting blood for these analyses two syringes should be used The first is to make careful venepuncture and to withdraw a few milliliters of blood, then the syringe is disconnected from

thrombin acts on the accelerator as outlined previously. It is only in human plasmas and similar specimens where the concentration of Ac-globulin is low that this is particularly noticeable. In such plasmas, the values are so low that the loss of a few units can be quickly noticed, whereas in the plasmas with higher values the loss of a few units does not affect the acceleration of the conversion of prothrombin to thrombin; and little loss can be detected.

As a word of caution, it may be remarked that this differential can be used only as an indication of some deficiency of Ac-globulin and will not serve in any but the crudest fashion as a quantitative test

In the determination of Ac-globulin a two stage analysis is used By diluting the original plasma many times to slow the conversion of prothrombin to thrombin, one can observe the rate of thrombin formation from purified prothrombin in the presence of thromboplastin, calcium and other accelerators By its action on a standard solution of fibrinogen, the amount of thrombin formed can be determined at repeated intervals As the amount of thrombin increases, the clotting time of thrombin-fibrinogen mixture will decrease Each of these determinations can be plotted and a curve representing the rate of thrombin formation can be constructed.

The rate of thrombin formation, with the other factors controlled, will be directly proportional to the Ac-globulin content of the plasma By altering the amount of Ac-globulin present a family of curves can be obtained. Such a group of curves is presented in the Figure 1 On the graph the intervals between the determinations of clotting time are represented on the horizontal axis in minutes The vertical axis represents the clotting time of the thrombin-fibrinogen mixture in seconds

By comparison of the curve obtained from analysis of an unknown plasma with such a set of standard curves, the level of Ac-globulin can be determined for the specimen An example of such a determination shall be given later

By using the incubation mixture to be described further on, the test for Ac-globulin is made more sensitive, particularly in man The sensitivity is increased by the reduction of the amount of calcium, and this will be found necessary when plasmas of low concentration of Ac-globulin are used By the increase in sensitivity, an increase in the dilutions can be made and thus the possibility of the formation of clot in the reaction tube will be lessened By the dilution, the reactive elements of coagulation are reduced to such a low level that coagulation can take place only at a very slow rate, slow enough so that there will be no interference with the thrombin formation that is being measured at regular intervals If the concentration of fibrinogen is high enough in the reaction mixture, fibrin formation will take place due to the constantly increasing production of thrombin The situa-

Separation and Preservation of Serum: Blood which is to be allowed to clot for the formation of serum can be drawn directly into a syringe and then transferred to a suitable tube for the formation of the clot. The tube is then placed in the ice box and full retraction is allowed to take place. This is usually complete after 2 hours At this time, the tube is removed from the ice box and centrifuged at 2000 g for 10 to 20 minutes. Serum can be preserved in the deep freeze (minus 10–20°F.) for a considerable length of time.

Preparation of Reagents

Thromboplastin A satisfactory preparation of thromboplastin for use in the two-stage analyses of prothrombin and Ac-globulin may be made as follows. Bovine lung, obtained at the slaughter house, is dissected free of the trachea, bronchi and the major vessels This is a gross dissection, the smaller vessels and bronchi remain in the lung. The lung is then ground through a domestic meat grinder and the ground material mixed with an equal amount of 0 85 per cent NaCl This mixture is then reground to insure complete mixing. The resulting suspension is then placed in the refrigerator and extracted for the next 24 hours Occasional stirring is necessary during this time. This is called the first extract. The next day the first extract is strained through gauze and discarded The remaining crude homogenate is then mixed with another equal volume of 0.85 per cent NaCl and replaced in the refrigerator for 48 hours Occasional stirring as before will be necessary. The second extract is then strained as before and then passed through a hand operated homogenizer (Central Scientific Co., Chicago, Ill.) At this stage it can be used for analyses Phenol is added to a final concentration of 0 5 per cent to prevent bacterial growth

The first extract is discarded because it will contain inhibitors of the conversion of prothrombin to thrombin as well as lacking in thromboplastic activity The second extract is considered to be purer and relatively free from antithrombic material. Third and fourth extracts have been tried and are low in thromboplastic activity

Before the second extract is mixed with phenol, it can be frozen and will remain stable for a long time For use, it is thawed and rehomogenized, because freezing seems to break the suspension After mixing with phenol, the thromboplastin should not be frozen. Apparently phenol disturbs the protein suspension and subsequent freezing will alter the proteins to such an extent that the material is no longer suitable for use

If the final lung suspension tends to flocculate or if there is a heavy precipitate or particles present in the final solution, it can be centrifuged lightly at not over 1000 g for about 5 or 10 minutes This removes the gross particles and does no harm to the thromboplastic preparation Re-

he needle and discarded, following which the second syringe is attached lirectly to the needle and the blood is drawn into the anticoagulant. Blood s then mixed thoroughly and is ready for centrifugation.

The choice of anticoagulant is important, particularly when the plasma s to be stored for any length of time. It is known that there is a loss in ome of the constituents of the clotting systems when blood is collected nto 1.85 per cent potassium or other oxalate salts This is particularly true or Ac-globulin. Therefore, a citrate solution, 3.2 per cent in physiologic aline is a better choice. Limited experience with heparin as a preservative, ndicates that it can be used, because storage after the use of heparin seems o be satisfactory.

When sodium citrate is used, one volume of the anticoagulant is added o nine volumes of blood; if potassium oxalate is employed seven volumes of blood are added to one of the oxalate.

The blood should be centrifuged as soon as is convenient after drawing, out generally this should not exceed one-half hour, although a longer delay s allowable, providing that the blood can be kept cool. If allowed to stand t room temperature, there may be considerable loss of Ac-globulin. Cenrifugation should be carried out at about 2000 g for about 20 minutes or onger in order to obtain adequate and complete separation of the plasma Some blood coagulation tests are profoundly influenced by the kind of entrifugation) If there is a slight amount of hemolysis after centrifugation, t can be disregarded. Experience has shown that this will be of little imortance in the evaluation of the clotting elements measured by the techiques described

Separation and Preservation of Plasma· After separation of the plasma, he analysis should be carried out as soon as possible. If, however, this is ot convenient, it can be stored for a few weeks in a deep freeze (minus 0–20°F) until the work can be done. Storage at these temperatures will naintain the plasma values for about a month. Longer storage is discourged due to the gradual loss of potency of some plasma components. Thawng and refreezing manipulations should be kept at a minimum, since this rocedure is detrimental to the proteins in the plasma. During the actual nalysis the plasma is best kept in an ice bath when not being manipulated, his will insure preservation of the plasma to the greatest degree

To thaw plasma that has been frozen, it is best placed in the water ath at 28°C for about 10 minutes with occasional agitation Even with his caution there may be some material in the plasma that will not dissolve This probably represents denatured protein that will go into solution with further heating or can be removed with a cotton swab on an appliator stick. Thawing at higher temperatures is acceptable if care is taken o prevent overheating

Imidazole Buffer: This is prepared by dissolving 1.75 grams of imidazole, (Edcan Laboratory, South Norwalk, Conn) in 90 ml of 0 1 N HCl and then diluted to 100 ml volume with distilled water. The pH can be adjusted with a few drops of strong hydrochloric acid or sodium hydroxide if it is not 7.2 or 7.4 which is the optimal range for the determinations and use of this buffer.

Fibrinogen· This protein, prepared by the method of Ware, Guest and Seegers, (page 227) is diluted to a 1 per cent solution with physiologic saline, and should contain 10 per cent imidazole buffer by volume It is stored in the deep freeze and thawed as needed. Care should be exercised during the thawing; rapid thawing usually being the most desirable. Slow thawing of the fibrinogen allows denaturation of the protein, which results in a reduction of the final concentration of the fibrinogen as well as being the source of insoluble material that will make clear definition of the endpoint difficult. If a method of rapid thawing is followed there will be little denatured material remaining Any of the altered material which does remain after the thawing can be removed with a small cotton swab on the end of an applicator stick. Different solutions of the fibrinogen will behave differently and the treatment should be altered according to each Change in the reactivity of this protein constitute one of the main sources of error in the determinations and infinite care is necessary

Steps in the Procedure· First step, *Dilution of the Prothrombin and the Ac-globulin* The dilution necessary for human plasma should be greater than 800 times for accurate curves to be obtained on the interpolation chart used in the calculation of the concentration Lower concentrations can be used but there are several disadvantages One of these disadvantages is that with the more concentrated solutions of plasma, there may be sufficient fibrinogen present, as well as other elements of the blood clotting mechanism, to cause coagulation in the reaction tube during the incubation period. There is no way to avoid this except by dilution, since the fibrinogen cannot be removed from the solution by any way known without altering the Ac-globulin or the other ingredients of the mixture

Another disadvantage is that with the increased concentration of the plasma there is an increase also in the plasma antithrombin, and this may destroy thrombin at a rapidly increasing rate, as the reaction produces more thrombin from the prothrombin The destruction of thrombin resulting, will manifest itself by a poorly shaped curve on the interpolation chart with a marked rise from the base line as the reaction proceeds This undesirable effect can be avoided by making the proper dilution of the plasma which will be a dilution large enough so that the effect will be so small that accuracy can be maintained

homogenation can be done at any time after the preparation has been made

Testing of the material is done by using it in the standard two-stage analysis for prothrombin using a known plasma as the substrate. It will usually be found necessary to dilute the thromboplastin to the ideal value A 1:4 dilution (before placing in the final reaction mixture) is commonly used, one part thromboplastin to four parts of 0 85 per cent NaCl. A less active solution may require a lower dilution and the reverse for a relatively active preparation.

Acacia and Calcium: Acacia is necessary in the incubation mixture to provide the proper colloidal environment for the reaction to progress In many respects it is a technical annoyance, but there is no easy way to dispense with it without throwing modern work out of gear with previously published data The type of acacia used is important only in that the amount of calcium contained must be known. We have obtained excellent results using crude acacia which has been analyzed and found to contain 0 68 per cent calcium. Purified acacia has been found to be no better than the crude. The crude acacia should be ground to a granular state and then dissolved in 0.85 per cent NaCl This procedure is slow and constant mechanical stirring is the best to obtain suspension of the material After the acacia has been dissolved it should be lightly centrifuged at about 2000 g for 5 minutes in order to remove the insoluble debris The material is then strained through 2 layers of gauze to remove particles that are not precipitated by the centrifugation It is then ready for use

A 15 per cent stock solution is prepared This concentration will provide the proper amount of calcium for the conversion of prothrombin to thrombin under the conditions of the reaction Since the amounts of calcium will vary from one batch of acacia to the next, the calcium will have to be determined with each new supply If any adjustments are required to obtain the final appropriate concentration of 0 70 per cent calcium, $CaCl_2$ is added until this is reached By keeping a large supply of acacia on hand in which the calcium content is known, these determinations will be avoided.

When this acacia is used in the Ac-globulin analysis there will be, when it is mixed with the thromboplastin, buffer and 0 85 per cent NaCl, the proper calcium concentration for the optimum performance of the test. The final concentration in the complete mixture has been determined as about 30 milligrams per cent, expressed as calcium In prothrombin analysis, the acacia solution in the incubation mixture will have to be strengthened with $CaCl_2$ until a final value of 65 milligrams per cent is obtained More calcium is required for the prothrombin assay then in the Ac-globulin determination.

solution should remain active and stable for several hours. However, after dilution, the material will lose activity in a shorter time and only as much as is needed for the work at hand should be prepared

Even with careful preparation prothrombin that is stored in the freezer at −5°C. gradually loses its strength until a plateau is reached, below which the level usually does not drop further for appreciable lengths of time.

There appears with the loss of the prothrombin, an accelerator of prothrombin conversion which seems to act in a similar manner as Ac-globulin It does not substitute for Ac-globulin although it acts in similar manner. This material appears in a few days in the dissolved prothrombin, but not in the dried powder. For this reason, only small amounts of the powder should be dissolved at any time and the prothrombin thus prepared used fairly quickly. When Ac-globulin determinations are done with this accelerator as a contaminant of the purified prothrombin, extremely high values are obtained When such occurs, the prothrombin should be discarded and a new preparation made. The use of a daily bovine plasma control enables one to guard against this potential pitfall

With the diluted plasma and the diluted prothrombin on hand, the final dilutions of the plasma can be made For example: if a final dilution of 1:800 is wanted for the plasma which contains about 300 units per ml of prothrombin, the first dilution should be 1·45 This yields plasma with a concentration of 6.7 units per ml This dilution is made with physiologic NaCl solution and is done by placing 0 1 ml of the plasma in 4 4 ml. of the solution. In these dilutions, as is the general practice, the pipet is washed out by sucking up the diluent several times Then 1 0 ml of this solution is placed in 3 4 ml of the dilute purified prothrombin, previously made up to 6.7 units per ml. Thus a final dilution of 198 is achieved, or 45 × 4 4. When this is further diluted with the incubation mixture in a ratio of 4.1 the final concentration of the plasma will be 4 × 198 or 792

This method of dilution is designed to be conservative of materials and will be the best in most instances Other methods of dilution are perfectly acceptable if the primary requirement that the concentration of the prothrombin is 6.7 units per ml in the final solution is met A thorough understanding of these dilutions is very important and necessary, before any familiarity with the analysis can be claimed

Second step, *Incubation Mixture* This incubation mixture, providing the other accelerators of the activation of prothrombin to thrombin, is composed of tissue thromboplastin, acacia, imidazole buffer and physiological NaCl solution It is prepared by mixing diluted thromboplastin with a prepared mixture of the other ingredients

The dilution of the thromboplastin is 1 5 with physiologic saline. This dilution is one that has been determined from experience as the most

This dilution of the plasma 800 times or more, may be done in one of two steps. In the former approach, a large amount of purified diluted prothrombin must be used while the second method will be more suitable when there is the desire to save materials. In the author's hands the second method is the better and will be described.

In designing the method of Ac-globulin analysis, Ware and Seegers chose a prothrombin concentration of 1.34 units per ml. which would give, on complete conversion to thrombin, a 12 second clotting time. The reason for this figure being chosen is that it shows on the interpolation chart the greatest change in value in the 13 to 17 second range. Examples and more complete elucidation of this will be shown later when the calculation of the results is explained

In order to obtain this level of prothrombin (1.34 units per ml.) in the final solutions, both the original plasma and the purified prothrombin used must be diluted to 6.7 units per ml This value is obtained when we consider that in the two-stage prothrombin test, in the conversion and thrombin measurement steps, there is a dilution factor of 5, and therefore the concentration of the final prothrombin is 5 × 1.34 or 6.7 units per ml. The prothrombin level of the original plasma should be determined and then diluted to the desired level with physiological NaCl solution and used for the test at that level. After this dilution, the plasma must be further diluted with purified prothrombin that has been previously adjusted to the desired 6 7 units per ml. This second step yields the final dilution of 800 or over.

The dilution of the purified prothrombin is made with physiological NaCl solution The prothrombin used is prepared from bovine plasma, heated at 54°C. for 1 hour and then acetone dried. The heating destroys any Ac-globulin that may be present For ease in handling, small amounts of this product can be dissolved in physiological NaCl solution and then placed in the deep freeze to await thawing at the convenience of the investigator

Products that have been freeze-dried are not as satisfactory for use in this analysis After such treatment there is a greater tendency toward the more rapid formation of thrombin than the acetone dried product and thus a reduction in stability. When choosing a product for use, one with highest practicable specific activity and purity should be selected, because there is a great loss in both, when the product is heated and dried. The final powder should be put into solution at about 670 units per ml for convenient handling in making the dilution necessary for use in the Ac-globulin analysis This concentrated solution is then frozen Thawing is best done rapidly in a water bath, care being taken that the prothrombin does not become heated and thus lose some of its activity After thawing, the concentrated prothrombin should be kept in an ice bath With these precautions the

The actual measurement of Ac-globulin concentration in the original plasma requires (a) interpolation from the standard curves of Ac-globulin activity, (b) correction for dilution, and (c) expression in terms of the control sample. To elaborate:

(a) Interpolation from the standard curves. If the procedure has been carried out properly, the curve constructed by connecting the points obtained by plotting the incubation time (horizontal axis in minutes) vs clotting time (vertical axis, in seconds) will have the same contour as the curves in figure 1. The data may read as in the following example:

Incubation time (min.).....	10	11	12	13	14	16	20	24
Clotting time (sec.).......	34	26	18.3	16	15	13.8	13.8	13.2

When these results are plotted on a chart such as in figure 1, they will be found to fall on the line labeled 16. Therefore, by definition, the conversion mixture contained 16 one-thousands of a unit of plasma Ac-globulin.

(b) Correction for dilution. The dilution preceding the activation is stated in steps 1 and 3 (pp. 203, 206) of this method and will be of the order of 800, as was calculated before. The dilution of the thrombin that occurs in the mixing with the fibrinogen is not a factor in the calculation, because Ac-globulin exerts its effect in the conversion of prothrombin to thrombin, and only the dilutions to this stage will affect the final activity of the Ac-globulin.

If we use the figure from the dilution example and the figure obtained from the example above, the final calculation is:

$$\frac{16}{1{,}000} \times 192 \times 4 = 12.8 \text{ units per ml of the original plasma sample}$$

(c) Relation to the control. The Ac-globulin test measures a dynamic process and for this reason is very sensitive to variations in the reagent activity. Therefore, when an unknown Ac-globulin sample is to be analyzed, a control should be done under the identical conditions and with the same reagents. This should be done simultaneously or within an hour of the determination of the unknown plasma sample. The normal control should be a bovine specimen in which the values are known to be stable.

If the control serum is needed for a serum Ac-globulin source, bovine blood is collected as before, but no anticoagulant is added. The blood is then allowed to clot for about two hours and the serum is separated from the cells by centrifugation. This serum is mixed with barium carbonate, on which prothrombin is preferentially adsorbed, followed by removal of the carbonate by centrifugation until clear serum is obtained.

The resulting serum will be practically prothrombin free and a potent source of serum Ac-globulin. It is stable and can be stored in the same manner as the control plasma.

satisfactory for use in the test; and after preparation of the thromboplastin from beef lung, it is diluted so that this five times dilution gives the proper answer in the standardization analysis.

The remaining materials, composing the incubation mixture, are added to the thromboplastin in a 2:1 ratio, one part thromboplastin and two of the mixture, as follows:

Acacia.. .. . . .2 parts (15% solution in 0 85% NaCl)
Imidazole buffer. .... 1 part (pH 7.25)
0 85% NaCl... .. ....3 parts

These materials are kept in the refrigerator all mixed, and a small amount of the mixture is used with the thromboplastin when an analysis is to be done. A convenient proportion will be 5 ml. of the diluted thromboplastin (1.0 ml. thromboplastin to 4.0 ml. of 0 85 per cent NaCl) and 10 0 ml. of the incubation mixture composed of the acacia, buffer and 0.85 per cent NaCl in the proportions described above. This final solution is placed in a water bath and kept at 28°C. as it is being used. There is a tendency for the thromboplastin to settle and agitation may be necessary.

Third step, *Activation*· To 2 1 ml of the reaction mixture, containing thromboplastin and the incubation mixture, is added 0 7 ml. of the diluted plasma containing 6 7 units per ml of prothrombin and diluted for its Ac-globulin content The mixture is placed in the water bath at 28°C and a stop watch is started at this time because this is the time when incubation begins, and prothrombin becomes activated

Fourth step, *Clotting of Fibrinogen to Measure the Thrombin Concentration* At measured intervals, usually two minutes apart, 0.3 ml of the conversion mixture is removed and added to 0 075 ml. of fibrinogen, previously pipetted into small, glass, 12 by 75 mm. tubes. The clotting endpoint, the same as that of the two-stage test for prothrombin, is determined accurately by means of a second stop watch, started at the instant of adding the conversion mixture to the fibrinogen.

The endpoint consists in seeing the first formation of definite granules in the solution observed at room temperature There is invariably a cloudiness developed in the previously clear solution as the endpoint is neared This is followed very shortly by the granularity. If this is the true endpoint, the granules are followed by the formation of fibrin strands and a definite clot in a few seconds A false endpoint, due often to variations in the acacia, is not followed by a clot, but usually remains granular for seconds

Calculations The plasma Ac-globulin unit may be defined as that amount of plasma Ac-globulin, which when diluted 1,000 times, will convert a standard solution of prothrombin (1.34 units per ml.) to thrombin under the conditions of the test, at the rate equivalent to line 1 in figure 1.

The actual measurement of Ac-globulin concentration in the original plasma requires (a) interpolation from the standard curves of Ac-globulin activity, (b) correction for dilution, and (c) expression in terms of the control sample. To elaborate:

(a) Interpolation from the standard curves If the procedure has been carried out properly, the curve constructed by connecting the points obtained by plotting the incubation time (horizontal axis in minutes) vs. clotting time (vertical axis, in seconds) will have the same contour as the curves in figure 1. The data may read as in the following example:

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Clotting time (sec.).	34	26	18 3	16	15	13 8	13 8	13 2

When these results are plotted on a chart such as in figure 1, they will be found to fall on the line labeled 16 Therefore, by definition, the conversion mixture contained 16 one-thousands of a unit of plasma Ac-globulin.

(b) Correction for dilution The dilution preceding the activation is stated in steps 1 and 3 (pp. 203, 206) of this method and will be of the order of 800, as was calculated before. The dilution of the thrombin that occurs in the mixing with the fibrinogen is not a factor in the calculation, because Ac-globulin exerts its effect in the conversion of prothrombin to thrombin, and only the dilutions to this stage will affect the final activity of the Ac-globulin

If we use the figure from the dilution example and the figure obtained from the example above, the final calculation is

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If the control serum is needed for a serum Ac-globulin source, bovine blood is collected as before, but no anticoagulant is added The blood is then allowed to clot for about two hours and the serum is separated from the cells by centrifugation This serum is mixed with barium carbonate, on which prothrombin is preferentially adsorbed, followed by removal of the carbonate by centrifugation until clear serum is obtained

The resulting serum will be practically prothrombin free and a potent source of serum Ac-globulin It is stable and can be stored in the same manner as the control plasma.

Stock supplies of these controls are kept in the deep freeze and thawed when they are needed. By the use of this bovine control, any change in the reagents can be detected, although it is desirable to use a control of the same species as the unknown, if this is possible. The control can be collected at the same time as the specimen for analysis or it can be frozen plasma that is of reasonably recent preparation. In some species the content of the Ac-globulin falls after the plasma has been placed in the deep freeze and stored for more than a short length of time.

Bovine material has a constant value of about 120 units per ml. and this value is used to determine the factor necessary to correct the values obtained if there is any deviation from this. A simple proportion is used For human plasma Ac-globulin values the range is 13 to 17 units per ml and 100 per cent has been placed at 15 units This is an arbitrary figure. Values obtained are expressed either as per cent of normal or in the actual units obtained from the determination, corrected for the difference shown by the control.

For example, in the specimen that we used above the answer was 12.8 units per ml If at the same time we had found that bovine control sample gives us 103 units per ml, the proportion would be as follows:

$$12.8 \times \frac{120}{103} = 15 \text{ units per ml for the specimen}$$

Other determinations done with the same reagents should be subjected to a similar correction.

Precautions and Sources of Error: A unit of thrombin is by definition that amount which will clot 1 ml of standard fibrinogen solution in 15 seconds, under standard conditions Clotting times between 13 and 17 seconds have been shown to relate the thrombin concentration most accurately One unit of prothrombin produces one unit of thrombin when fully converted. In this test the amount of prothrombin, 1.34 units per ml, is such that full advantage is taken of the 13 to 17 second range of end-points As a result all curves indicating Ac-globulin concentration down to line # 12 of figure 1 have the principal change of direction from vertical to horizontal in the 13 to 18 second range

Although the prothrombin concentration is arranged so the complete conversion furnished 1 34 units per ml, the actual final thrombin yield in the Ac-globulin test is dependent on the Ac-globulin present Comparison of the curves # 64 and # 32 in figure 1 show that the former ultimately gives 12.0 second clotting whereas the latter only reaches 12 8 seconds Reducing the Ac-globulin further continues to reduce the final thrombin yield

Occasionally the form of the experimental Ac-globulin curve does not fit into the contours imposed by the interpolation chart (fig 1) In such

instances it may cross one of the curves which are a part of the interpolation chart Often the defect is of the nature illustrated by line A of figure 1, crossing to the less concentrated side and leveling off shallow. Such a pattern may be caused by a deficiency of prothrombin in the conversion mixture, the presence of serum type Ac-globulin in the analytic sample, low fibrinogen activity, abnormal colloid forms or calcium concentration. Any of the rest of the reagents in the solutions may be suspected as well, although those mentioned will be the most likely sources of the trouble. Canine plasma gives this sort of curve normally and they are most difficult to interpret on this account.

Conversely, a poor curve of the opposite form may pass as does line B of figure 1, to higher values, leveling off in the region below that expected from the location of the upper portion of the curve, perhaps even with endpoints that are below the 12 0 second line. Excessive amounts of prothrombin or hypersensitive fibrinogen are usually responsible for this type of curve. Should the curve remain abnormal after checking the prothrombin activity of the plasma, the diluted purified prothrombin, and the fibrinogen, then the other materials must be checked as above.

Successful utilization of this test requires strict control over the reagents both for the determination of prothrombin and Ac-globulin. To be regarded as fully satisfactory they should give when used in testing human

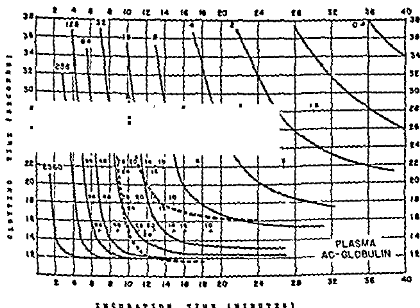


Fig 2

or bovine plasma: (1) Bovine or human prothrombin values by the modified two-stage method of 250–300 units per ml. (2) Plasma Ac-globulin curves with good contour, for normal bovine plasma, diluted 3000–5000 times, in the range 32–128 on the interpolation chart. (3) Plasma Ac-globulin curves with good contour, for human plasma diluted 1000 times, in the range of 12–48 on the chart.

Multiple Analyses. When the reagents are standardized several Ac-globulin tests may be run at the same time. By preparing the fibrinogen ahead of time it will be possible, by utilizing the latent period of each test (this is the time intervening after the mixing of the conversion mixture and the diluted plasma; before there is any detectable amount of thrombin formed), to set up new determinations at two-and-one-half minute intervals. If this is done, five determinations can be done at the same time. This will enable the investigator to run a control simultaneously with four plasmas. However, the number of points that can be plotted will be reduced due to the overlap of some of the dilutions but since this is a determination of rate and curves are used in the calculation of the answer, this will still be accurate for most of the work to be done With fewer tests closer intervals of time between determinations of clotting time can be done if greater accuracy is desired.

Serum Ac-globulin analysis. Ac-globulin, when detectable in serum, give a different type of curve than that shown by plasma Ac-globulin. Conditions for testing for the serum Ac-globulin are the same as those for the plasma type. A special set of quantitative curves are, however, necessary for the final interpolation These are illustrated in figure 2. The difference in the curves given for the plasma type and the serum type Ac-globulin is attributed to the fact that the former is activated along with thrombin formation causing an initially greater delay in thrombin appearance, but more rapid prothrombin conversion when finally under way, whereas the latter is already active when the conversion of prothrombin is begun.

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3. Concentration of Bovine Ac-Globulin

W. H. SEEGERS and N. AOKI

Ac-globulin is one of the unique plasma proteins of great importance for the activation of prothrombin In the presence of this protein, thrombin and autoprothrombin C are altogether different enzymes. With thrombin alone, prothrombin activates mainly to autoprothrombin II, but if Ac-globulin is also there, thrombin is the chief activation product. With autoprothrombin C alone, prothrombin activates mainly to autoprothrombin. Ac-globulin changes the enzyme substrate specificity and the thrombin generates from prothrombin in the presence of autoprothrombin C.

Procedure: Fresh bovine blood is mixed with a 1.85 per cent potassium oxalate solution in the proportion of 9 to 1 respectively, and the plasma is separated by centrifugation, used immediately, or frozen and used within several weeks. To remove prothrombin from the plasma, 1 liter of bovine plasma is mixed with 40 Gm. of barium carbonate and stirred for 10 minutes. The barium carbonate is removed by centrifugation and this adsorption procedure is repeated Next, the plasma is diluted with 5 liters of cold distilled water and brought to pH 6.1-6.2 with the addition of 1 per cent acetic acid while being mechanically stirred at 0°C The precipitate is removed by centrifugation in the cold and discarded It contains large amounts of fibrinogen and inert protein The supernatant solution keeps almost all the activity of the original plasma and the active material is precipitated by adding more acetic acid at 0°C until the pH is 5.0-5 1. The precipitate is sedimentated by centrifugation in the cold at 2000 g for 10 minutes. The sides of the centrifuge tubes are rinsed with a stream of cold distilled water and then the sediment is homogenized and suspended in a liter of cold buffered saline which contains 30 ml 0 05 M pH 7 0 phosphate buffer The pH of the solution becomes 6 8 This solution contains about 70 per cent of the activity of the original plasma

The next step consists of fractionation with ammonium sulfate Two hundred and eighty-two Gm. of solid ammonium sulfate are added to the solution which is kept at −2°C. with an ice-salt bath while mechanically stirring. This gives a concentration of about 45 per cent saturation After all of the solid ammonium sulfate is added, the solution is stirred continuously for a few minutes while the temperature is kept near −2°C. The precipitate is collected by centrifugation at 0°C at 2500 g for 15 minutes. The supernatant solution, which contains about 80 per cent of the protein but only a little activity, is discarded. The precipitate usually has more than 50 per cent of the activity of original plasma This is dissolved with

a minimum volume (perhaps 20 ml) of distilled water and dialyzed against cold water. The pH of the water used for dialysis is treated by adding 1 ml. saturated sodium carbonate solution to 8 liters of the distilled water. Without this, quite a loss of activity commonly occurs. An efficient dialysis apparatus is used. The water is changed 4 times so that the dialysis is completed in 2 hours. The specific resistance after dialysis is 1000 ohms more or less, and the pH is around 7.

When the desired resistance is reached, the solution is put in a beaker and brought to pH 6.3-6 4 by addition of 1 per cent acetic acid dropwise, with constant stirring and with the temperature near 0°C. The solution is centrifuged in the cold at 2000 g for 10 minutes. The precipitate, consisting mostly of inert protein, is discarded. Then more acid is added to the supernatant solution in the same manner as before with constant stirring until the pH is between 5.2 and 5.3. The precipitate is sedimentated by centrifugation in the cold at 2000 g for 10 minutes. The supernatant solution is discarded, the sediment is broken up completely with a glass rod, and about 4 ml. of saline is added gradually. The saline is buffered with 0 2 volume of 0 05 M phosphate buffer, pH 7.0. The Ac-globulin concentration procedure described can easily be carried through by one person in about 6 to 7 hours.

The optimum pH for best stability is found to be 7.0. There is a definite tendency for the activity to fall off as the acidity or alkalinity is increased. One unit of pH either way is already quite significant.

In physiologic saline solution, buffered at pH 7.0 with imidazole, full activity is retained for several hours at room temperature, in a refrigerator for 2 days, and in a deep freeze for 7 days. In the same conditions as above, but also with glycerol added to a concentration of 50 per cent, all the activity is retained at room temperature for 1 day, in the refrigerator 4 days, and in a deep freezer for more than 3 weeks. We also obtained some information on the usefulness of calcium as a stabilizing agent. In a mixture at pH 7, 50 per cent glycerol, and 0 08 M calcium chloride, full activity is retained at room temperature for 2 days. At −60°C, all activity is there at the end of 1 month.

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4. Determinations of Factor VII (Proconvertin)*

L. PECHET

General Considerations. Serum derived from blood which undergoes spontaneous coagulation contains two (or more) entities which accelerate the conversion of factor II (prothrombin) to thrombin, namely factor VII† (proconvertin)[1] and factor X (Stuart)[2] Factor VII is activated from a relatively inert precursor during coagulation,[1] by proteolytic enzymes[3] or by thromboplastin-calcium[4]

For the determination of factor VII it is essential that all the factors required in the prothrombin conversion mechanism, except for factor VII, be provided in optimal amounts. Since factor VII acts via the thromboplastin-Ca-prothrombin conversion mechanism, it can be measured only by its effect on the conversion of prothrombin to thrombin In its absence, prothrombin conversion is markedly retarded, the Quick prothrombin time thus being prolonged. The addition of factor VII to a plasma congenitally deficient in it, or otherwise rendered deficient, accelerates coagulation and shortens the prothrombin time Since factor X has properties very similar to those of factor VII (both are adsorbed by the same adsorbants, and depressed by dicoumarol), plasma *rendered* deficient in factor VII will also be deficient in factor X, and the methods using such plasma as a substrate will be sensitive to both factors, referred to sometimes as "factor VII complex." On the other hand, if plasma from subjects with known absence of one of these factors is used as substrate, the results are specific. A method for factor X which obviates this difficulty uses Stypven as a reagent, and is described in Chapter VI, Section 14 (p 129)

Two methods for measuring factor VII are described (a) the method of Owren,[5] sensitive to both factors VII and X, (b) a method for the specific determination of factor VII, using plasma congenitally deficient in factor VII

A *Determination of Factor VII (Proconvertin) by the Method of Owren*

Principle The procedure is essentially a one-stage prothrombin method, employing a technic similar to the one described for the specific determination of factor II (p 144) The test mixture comprises bovine oxalated plasma rendered devoid of factors VII and X by filtration through a Seitz pad consisting of 20 per cent asbestos Such a plasma, rich in

* The present text is based partially on the method previously described by Dr. Benjamin Alexander in Chap VIII of the first edition of this book

† Described in the first edition of this book as SPCA.

factors II (fibrinogen) and V (Ac-globulin), also contains 60 per cent of original factor II (prothrombin), yet has a markedly retarded prothrombin conversion.

Reagents and Apparatus are similar to those described for the specific determination of factor II (p. 144).

Seitz-filtered bovine oxalated plasma. Freshly shed bovine blood, obtained at the slaughterhouse, is collected on 0.15 M potassium oxalate as described on page 146. The plasma is then filtered slowly under positive pressure through a 20 per cent asbestos Seitz pad.* Only those samples are used which when combined have a prothrombin concentration of 60 per cent of normal or more and a Quick prothrombin time of approximately 180 seconds. The filtered plasma is subdivided into aliquots, each of sufficient volume for 1 day's supply These are kept in the frozen state (−15 to −20°C.); under such conditions the material is stable for at least 6 months.

Performance of the Test. The test material is suitably diluted with VBOS.† One-tenth ml of the diluted material is added to 0 1 ml of substrate (the bovine filtered plasma). The mixture is allowed to come to bath temperature (37°C), then 0.2 ml. of the calcium-thromboplastin reagent are added, and the clotting time is measured

Values and Calculations All determinations are expressed in terms of the activity of normal plasma The plasma precursor of factor VII becomes activated by thromboplastin and calcium, and then acts on factor II in the presence of factor V and calcium. A standardization curve for the particular substrate and thromboplastin used is derived from the observed effects of normal pooled (from at least five subjects) human oxalated plasma upon the prothrombin time of the substrate rich in prothrombin but devoid of factor VII by Seitz filtration The effect of a 1 to 10 dilution of the normal plasma with VBOS is taken as the 100 per cent standard activity, further subdilutions of this giving correspondingly lower values The observed clotting times are plotted against the per cent activity (fig 1) on a log/log graph

The factor VII activity of a test material, also appropriately diluted with VBOS, is computed by interpolating the observed clotting time on the standardization curve In 73 normal subjects studied in 1 day in our labo-

* Obtained from Carlson, Ltd , London, England The speed of filtration and the volume filtered in relation to the size of the pad are important variables, determined by trial and error, in obtaining the desired result One pad, 14 cm in diameter, is used to filter a vol of 400 ml of plasma at a rate of 80 drops per minute The first 80 ml , if devoid of both factors II and VII, are discarded The next portions are usually rich in factor II but devoid of factors VII and X The material is collected in 25 ml aliquots

† At least ten-fold.

ratory, the range for factor VII complex was 78 to 170 per cent (± 2 standard deviations on a logarithmic scale) with a mean of 115 per cent.

Limitation: This test does not distinguish between factors VII and X. When found abnormal, further testing is necessary in order to distinguish which one of the two entities, if not both, is depressed (or elevated).

Precautions and Sources of Error: Precautions in the drawing of the blood samples are essential for this method. Normal serum contains an appreciable fraction (approximately 10-20 per cent) of factor VII in the activated form and exhibits considerably greater activity than the corresponding plasma from which it was derived. Thus, anything which favors factor VII activation (contamination of needle and syringe with tissue juice from inept venepuncture, from agitation of the blood, from excessive air-blood interface, etc.) will give higher values.

If the method is used for serum determinations, the control curve should be built with normal pooled serum (fig. 1).

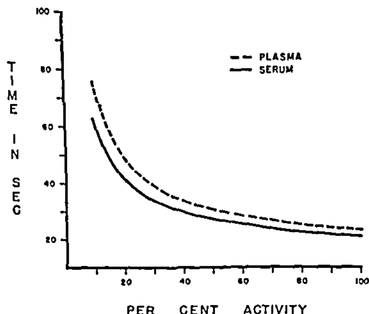


FIG. 1.—Typical standardization curve, correlating clotting time with activity. Pooled normal human oxalated plasma (or serum) added in various proportions to bovine plasma filtered through a 20 per cent asbestos Seitz pad. One hundred per cent activity corresponds to 0.10 ml. of a 1:10 dilution of normal plasma with VBOS, added to 0.1 ml. of the bovine plasma. To this are added 0.2 ml. of the thromboplastin-calcium reagent. Note the relatively greater activity of equivalent amounts of serum compared with plasma.

It is also important that the prothrombin content of the bovine-filtered plasma be at least 60 per cent of normal. Under such conditions, the addition of prothrombin in the test material (plasma, serum, or fractions), which has been suitably diluted with VBOS prior to addition, introduces a negligible increase in the prothrombin concentration of the total system If the bovine plasma reagent gives a Quick prothrombin time in excess of 180 seconds and yet contains more than 60 per cent factor II, one may be assured that the reagent is virtually devoid of factor VII complex

B. *Determination of Factor VII (Proconvertin) by a One-Stage Specific Method.*

Principle This test is based on correction of the Quick one-stage prothrombin time, abnormally prolonged in plasma congenitally deficient in factor VII, but normal in every other factor, as established by individual specific tests Plasma from a patient previously described,[6] or from dogs congenitally deficient in factor VII,[7] generously provided by Dr J. F. Mustard, was used. To this, $BaSO_4$-absorbed bovine plasma is added in order to restore factor V, partially lost by freezing or lyophilization of the substrate plasma Upon addition of the material to be tested, the prolonged Quick prothrombin time is shortened, depending exclusively on the amount of factor VII contained in this material. Unlike Owren's method, this test is not influenced by factor X.

Reagents and Apparatus

a Same as for the Quick one-stage prothrombin determination, plus: Oxalated human or dog plasma, congenitally deficient in factor VII, and kept frozen (−15 to −20°C), or lyophilized.

b $BaSO_4$-absorbed bovine plasma prepared according to the procedure described for the specific determination of factor II (p 214).

Performance of the Test To 0 1 ml. human or rabbit brain thromboplastin, the following reagents are added in order,

0 05 ml barium sulfate adsorbed bovine plasma,
0 025 ml. substrate (plasma deficient in factor VII),
0 025 ml. of the material to be tested (or dilutions of the normal pooled plasma from which a curve is obtained),
0.1 ml calcium chloride, 0 02 M, which is kept in the water bath during the performance of the test

A stop watch is started simultaneously with the addition of the $CaCl_2$ The clotting time is determined, as in the Quick method, either by using a nichrome wire loop in a glass water bath, or by the tilt method

In order to construct a calibration curve, normal pooled human plasma is used Dilutions with saline to 50, 20, 10, 5 and 1 per cent are made. The clotting times are plotted on regular graph paper as for the Quick method and the curve obtained The clotting time range obtained usually for both human and canine plasma substrate is described in figure 2

In 63 normal subjects studied in 1 day in our laboratory, the range for factor VII was 58 to 164 per cent (± 2 standard deviations on a logarithmic scale), with a mean of 97 per cent.

Precautions and Sources of Error. The most sensitive part of the curve is between 20 and 5 per cent. It is, therefore, desirable to dilute the material to be tested as to give a clotting time within this range of the curve. A curve must be prepared every day the test is performed In order to avoid activation of factor VII by freezing, a fresh pool must be obtained every time a curve is built. Each point on the curve and the clotting time of the unknown sample is obtained by duplicate determinations which should agree within 1 second. The collection of blood, that of bovine plasma, and the absorption on $BaSO_4$ must be performed with the same precautions described for Owren's methods for factors II and VII. The substitute should contain less than 1 per cent Factor VII.

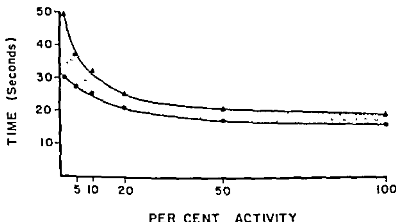


FIG 2.—Standardization curve for the specific one-stage factor VII (proconvertin) determination Range indicates clotting times obtained for each dilution of the plasma pool on different occasions using either human or dog plasma congenitally deficient in factor VII only

Acknowledgment

The author wishes to acknowledge the help of Miss Frances Cochios in preparing this manuscript.

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CHAPTER IX

FIBRIN AND PRECURSORS

1. *Estimation of Plasma Fibrinogen (Rapid Method of Schneider)*

Adapted by L. M. TOCANTINS

Principle. Blood is diluted and the highest dilution in which visible coagulation occurs when thrombin is added is termed the "fibrin titer."

Reagents and Apparatus: (a) Glass tubes 12 x 100 mm (b) Ringer's solution (or alternatively a solution made up of 9 parts 0.85 per cent NaCl and 1 part of 1/40 M $CaCl_2$) (c) Thrombin (bovine "topical thrombin"): dissolve the contents of 1 vial in enough 50 per cent glycerol to make a solution of 100 units per ml This keeps well in the refrigerator for months

Steps in the Procedure

Arrange two rows of tubes, 8 in each row Label one "patient," the other "normal control."

Dilutions: First place 3 ml. of the diluent in the second tube of each row of tubes, 4 ml. in the third tube, and 1 ml in each of the remaining tubes

Venous blood is then drawn from the patient into a syringe of small diameter (a 1 ml , or ½ ml tuberculin syringe or insulin syringe is of small enough diameter to be admitted deeply into the dilution tubes for mixing and sampling) and is dispensed and diluted, at once, directly from the syringe, at the beside, into the series of assay tubes in the following manner. An undiluted control sample is set aside by discharging all but 0 5 ml of the blood from the syringe into the first tube The remaining 0 5 ml of whole blood is then discharged *into the second tube* and is thoroughly mixed with the 3 ml of diluent already in it With the same syringe, 1 ml of this mixture is carried over to the next tube The remaining twofold dilutions are accomplished by carrying over a 1 ml aliquot serially from each tube to the next Assuming a hematocrit of approximately 0 35, the resultant dilutions of the plasma component are 1, 10, 50, 100, 200, 400, 800, 1600

With care, the original syringe may be used to make the dilutions. In each successive dilution, the mixing may be accomplished by repeated aspiration and ejection from the syringe. During this mixing process, the plunger of the syringe itself may be removed and immersed into the diluted mixture once before the final fluxing; this reduces the risk of excessive "carry-over" from one dilution to the next, particularly during the first dilutions. To each tube is added 10 units of thrombin (0.1 ml. of the solution from a pipette, or 1 drop from a dropper). Mix well. Coagulation occurs in a few seconds and is complete in a few minutes.

Readings: The rack of tubes is allowed to stand at room temperature, preferably without shaking, until the time of reading. Each tube is then tilted back and forth to find the highest dilution in which a visible coagulum has formed. In the dilutions near the end point, the fibrin may contract to a minute clot, or, indeed, if there has been shaking, may accumulate directly as a minute clot.

Control: Because of the infrequency with which one may use the fibrin titer assay, it is desirable to perform a control titer at the same time, on the blood of one or more normal pregnant women, near term or just delivered, to provide a check on the technic. When several such comparisons are made, it is found that a difference of one tube is not necessarily significant.

A control sample from a nonpregnant individual may be used if it is kept in mind that the fibrinogen content is approximately 250 mg. per cent rather than the usual 500 mg. per cent that is more characteristic of normal term pregnancy; still higher levels may occur in toxemia of pregnancy.

A titer of 800 is commonly found in normal pregnant women near term Variations in the "titer" assay are usually limited to one tube, in either direction, by observation of careful technic of dilution.

Reference

Schneider, C L · Rapid estimation of plasma fibrinogen concentration and its use as a guide to therapy of intravascular defibrination Am J. Obst. & Gynec 64, 141, 1952

2. Plasma Fibrinogen Titer ("Thrombin Titer")

H. S. BOWMAN

Object of the Test: Rapid estimation of the effective fibrinogen content of plasma.

Principle: The reciprocal of the highest saline dilution of citrated plasma in which a visible fibrin clot occurs after addition of thrombin is termed either the plasma fibrinogen titer, or "thrombin titer."

Preparation of Reagents and Apparatus:

(1) *Test plasma:* Five ml. of fresh venous blood drawn from the patient is placed into a collection tube containing 0.1 ml. 19 per cent sodium citrate. The specimen is centrifuged at 1200-1500 g to obtain the supernatant platelet-poor plasma. Other anticoagulants may be used but might yield lowered titration values. EDTA plasma in which divalent cations are unavailable should be avoided

(2) *Thrombin:* Fifty units of human thrombin (Fibrindex, Ortho) are dissolved in 0.5 ml. normal saline, and used as a fresh preparation only Alternatively, employing sterile equipment, 1000 units of bovine "topical" thrombin are reconstituted with 5 ml. distilled water and added to a vial containing 5 ml. CP glycerol The bovine thrombin-glycerol solution when stored at 4°C. may be used for repeated assays over a 1-month interim. The thrombin solutions are well mixed, resulting in a concentration of 10 thrombin units/0.1 ml.

(3) *Normal saline,* 0 85 per cent sterile solution

(4) *Glass clotting tubes,* 13 x 100 mm., and rack

(5) *Serologic pipettes,* 5 ml and 1 ml.

Steps in the Procedure: Arrange a series of seven test tubes, 13 x 100 mm Pipette no saline into the first tube, 4 5 ml. saline into the second tube, 0 5 ml saline into the third tube, 2 0 ml saline into the fourth tube, and 1.0 ml. saline into the fifth, sixth, and seventh tubes.

Place 0.5 ml of the test plasma into the first tube, and another 0 5 ml of the test plasma into the second tube With a fresh pipette, mix the contents of the second tube by drawing up and discharging 6 times with the pipette, then discard the pipette

With a fresh pipette, transfer 0.5 ml of the plasma mixture from tube #2 to tube #3. Also transfer an additional 0 5 ml of the plasma mixture from tube #2 to tube #4. Mix the contents of tube #3 well and discard the pipette.

With a fresh pipette, mix the contents of tube #4 well, then transfer 1.0 ml. of its plasma mixture to tube #5. Mix well by drawing up and discharging 6 times with the pipette, and in a similar way transfer 1.0 ml of the plasma dilution in tube #5 to tube #6, and 1.0 ml. of the plasma dilution of tube #6 to tube #7. Discard the final 1.0 ml. residual from tube #7 Avoid air bubbles in preparing dilutions.

With a sterile pipette and at room temperature, add 0.1 ml. of thrombin solution to each tube. Shake each tube lightly to mix. Let stand for minimum of 90 seconds.

Readings: Examine each tube by tilting gently in a horizontal position against a good light source. In tube #1 a firm fibrin clot and in tube #2 a gelatinous fibrin clot normally form that adhere when these tubes are inverted A transparent white pellicle forms in the remaining tubes When the end point is in tube #6 or #7, the fibrin clot is small, filmy, and often attached to the meniscus at the top of the plasma-saline mixture. The highest dilution in which a visible *single* coagulum of fibrin is seen is the fibrinogen or "thrombin" titer. Reciprocals of the plasma dilutions are. tube #1: 1; tube #2: 10; tube #3: 20; tube #4: 50; tube #5: 100; tube #6: 200; tube #7: 400.

Normal Values The normal range is 100 to 400 In pregnant women the mode is 400, and in normal non-pregnant women and men the mode is 200. In significant fibrinogenopenia, the soft clots occurring in tubes #1 and #2 are non-adherent and usually slide down the tube upon inversion. A normal test is illustrated in figure 1.

Precautions and Sources of Error in Fibrinogenopenia This modification of Schneider's original test[1] utilizes the more accurate plasma dilutions provided within the laboratory rather than dilution of whole blood at the bedside, and as its normal limits are defined[2] no control is required. The proportion of 19 per cent sodium citrate to whole blood furnishes a 97 per cent plasma concentration Other anticoagulants, as 0.1 M sodium oxalate, may yield an initial 83 per cent plasma concentration and artificially dilute the plasma fibrinogen prior to testing

This determination is most useful in evaluating defibrination syndromes,[1,3] in which orthodox biochemical methods (micro-Kjeldahl) or rapid ammonium sulphate precipitation may not suggest dangerously low levels of fibrinogen that exist In such disorders the circulating plasma fibrinogen may be imperfectly reactive to thrombin as a consequence of the coagulation defect, and most reliably detected by this procedure.[3]

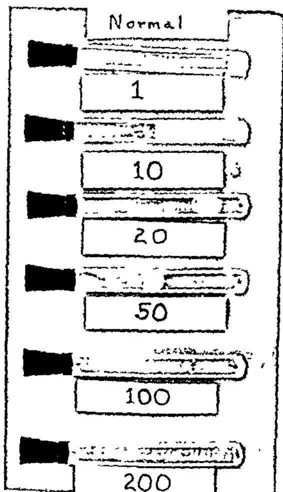


FIG 1—A normal plasma fibrinogen titer. In dilutions 1 and 10 there is a solid fibrin clot that adheres to the wall of the tube The end point is 100

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3. Estimation of Fibrinogen in Small Samples of Plasma

O. D. RATNOFF and C. MENZIE

Principle: The amount of fibrinogen in plasma is determined by converting it to fibrin in the presence of crushed glass [1] The fibrin adheres to the glass and can be quantified by measuring its tyrosine content with Folin-Ciocalteu phenol reagent.

Reagents and Apparatus

(1) Plasma. Glass or Lusteroid centrifuge tubes, approximately 13 x 100 mm in size, are prepared by pipetting into each 0.5 ml. of a solution containing 0.8 Gm of potassium oxalate and 1.2 Gm. of ammonium oxalate per 100 ml. The anticoagulant solution is dried before use. Five ml of venous blood are transferred immediately after withdrawal to a tube containing the dried anticoagulant salts. The plasma is separated by centrifugation, for example, for 10 minutes at 2000 rpm in a Sorvall angle centrifuge. For routine testing, the plasma is either assayed immediately or frozen at −25°C. until a suitable time

(2) Crushed glass Pyrex glass is crushed in a mortar until the diameter of the largest particles is approximately 0.5 mm., and used without further grading or removal of glass dust Between each use, the glass particles are washed with chromic acid, rinsed and thoroughly dried. Crushed glass may also be obtained commercially.

(3) Sodium chloride solution, 0.85 per cent.

(4) Thrombin. Commercial bovine "topical" thrombin, containing 1000 N.I H. units per ml. This can be frozen at −25°C. for several weeks without affecting the assay

(5) Sodium hydroxide solution, 10 Gm. per 100 ml. of aqueous solution

(6) Sodium carbonate solution, 20 Gm. per 100 ml. of aqueous solution.

(7) Folin-Ciocalteu phenol reagent (Fisher Scientific Co or Hartmann Leddon Co., Inc.)

(8) Tyrosine standard solution 200 mg tyrosine per liter of 0.1 N hydrochloric acid (Hartman Leddon Co., Inc , Philadelphia).

(9) B-D Cornwall Luer-Lock automatic syringe, 10 ml., used for all additions of 0.85 per cent sodium chloride solution, for speed.

(10) Beckman Model B spectrophotometer or equivalent.

Steps in the Performance of the Test: To a 40 ml round-bottom Pyrex centrifuge tube are added approximately 0 5 ml of crushed glass, 10 ml of 0.85 per cent NaCl solution and 0.05 ml. of the thrombin solution Then 0 5 ml. plasma are pipetted into the tube, which is agitated with an oscillatory motion Volumes of plasma as small as 0.1 ml. may be used As clotting occurs, the fibrin adheres to the glass particles. With active preparations of thrombin, clotting is usually complete within 2 to 3 minutes If clotting continues after the cessation of shaking, further agitation and rotation results in the adherence of the new clot to the glass After 10 minutes the tube is centrifuged for 5 minutes at 2000 rpm in an angle centrifuge. The supernatant is discarded. The clot is washed twice by adding 10 ml. of the 0 85 per cent NaCl solution to the tube, and recentrifuging for 3 minutes. The wash solutions are discarded. One ml. of 10 per cent NaOH is then added to the tube and the mixture is heated in a boiling water bath for 10 minutes Evaporation is minimized by covering each tube with aluminum foil. After the tube cools, 7 ml of water are added, followed by 3 ml. of sodium carbonate solution and 1 ml. of Folin-Ciocalteu reagent. *For accurate results, the tube must be swirled rapidly after the addition of the sodium carbonate solution and again after the addition of Folin-Ciocalteu solution.* The blue color is fully developed within 10 minutes; if the crushed glass is omitted, 30 minutes are required.

The intensity of the color is then read in 10 mm cuvettes in the Beckman Model B spectrophotometer at a wave length of 650 mμ If a Coleman Junior spectrophotometer is used, the solution must be diluted with 3 volumes of water prior to reading A blank solution is prepared in the same fashion, except for the addition of plasma; this blank solution must be heated in the boiling water bath, or inaccurate results will be obtained

Standard Solutions. Two standard solutions are prepared by the addition of 0.5 or 1.0 ml. of tyrosine solution to a 40 ml centrifuge tube, and, in succession, 1.0 ml. NaOH solution, 6 5 or 6 0 ml. of water respectively, 3 0 ml. sodium carbonate solution and 1 0 ml. of Folin-Ciocalteu reagent. After 10 minutes, the intensity of the blue color is read in the spectrophotometer against a blank containing 7.0 ml water, 1 0 ml NaOH solution, 3 0 ml sodium carbonate and 1.0 ml Folin-Ciocalteu reagent. Neither the standard solution nor this blank are heated Note that two separate blank solutions are prepared, for comparison with the heated fibrin solution and with the standard solution respectively

Calculation· The amount of fibrinogen in the aliquot tested is calculated by multiplying its tyrosine-like activity by 11 7 The tyrosine-like activity of the aliquot is calculated by arithmetic proportion by comparing its optical density with that of a standard solution of tyrosine. The standard solution

whose optical density more closely approximates that of the unknown is used in this calculation. Finally, the fibrinogen content of the sample is multiplied by whatever factor is needed to express the result as mg per 100 ml. of plasma. For example, if the O.D. of a 0.5 ml sample is 0.500, and the O.D. of the 100 μgm. tyrosine standard is 0.550, then

$$\frac{0.500}{0.550} \times 11.7 \text{ (conversion factor)} \times 200 = 213 \text{ mg. fibrinogen/100 ml}$$
plasma.

Normal Range of Values: In 41 normal men, the fibrinogen content of the plasma varied from 177 to 415 mg. per 100 ml., averaging 265 mg. per 100 ml. of plasma. In 36 normal, non-pregnant women, the fibrinogen content varies from 164 to 485 mg per 100 ml., averaging 294 mg The difference between the concentration of fibrinogen in men and women is not significant.

Precautions and Sources of Error. All determinations are performed in duplicate; with samples as small as 0.1 ml., duplicate values should agree within 10 per cent. A small error may be introduced into the determination of plasma fibrinogen by the adsorption of other proteins onto the fibrin clot. Uncommonly, the formation of fibrin may be slow or incomplete, perhaps because the plasma possesses anticoagulant activity. In such cases, 0 5 or 1.0 ml of thrombin may be used; this amount is not used routinely, because occlusion of thrombin into the clot may give falsely high values. In fibrinolytic states, the incorporation of epsilon aminocaproic acid into the assay mixture before the addition of thrombin has been suggested to prevent fibrinolysis prior to the digestion of the clot [2] Our own experience is too limited to permit evaluation of this step.

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4. Preparation of Fibrinogen

J. F. JOHNSON and W. H. SEEGERS

In analytic experiments it is essential that there be a constant source of active, purified fibrinogen in order to insure accuracy and reproducible results Commercial preparations, if such are available, are usually not adequate, containing only from 75 to 90 per cent clottable protein. A simple method to obtain fibrinogen, based upon the observation that fibrinogen is not completely soluble in cold plasma, is here described. This method, carefully carried out, regularly yields a product which, after freezing and thawing, remains clear for many hours and is "free" of prothrombin, fibrinolytic enzymes, and other elements concerned in the clotting reaction It remains stable and maintains its reactivity for many months in the deep freeze.

Materials and Reagents: (a) Plasma. The blood used should be collected in a special anticoagulant (1.85 per cent $K_2C_2O_4 \cdot 2H_2O$ and 0 5 per cent $H_2C_2O_4 \cdot 2H_2O$), because the resulting plasma has a low salt concentration. The usual procedure is to collect the blood at the slaughter house. The plasma is separated from the blood by centrifugation This plasma is then frozen solid at deep freeze temperatures

(b) Saline. 0 85 per cent NaCl is used throughout This has either been pre-cooled to 0°C or has been placed in the cold overnight where it may even be frozen and then used at it thaws. A suitable buffer for use in the dilution of fibrinogen is prepared by dissolving 1 72 grams imidazole in 90 ml of 0 1 N HCl and then diluting with distilled water to 100 ml. The final solution will be of a pH of 7 2 to 7 4. If this is not achieved, the end point can be adjusted with a few drops of NaOH or HCl

Steps in Procedure About four gallons of the frozen plasma is broken into small chunks and force-thawed in a metal container An electric fan directed at the container will aid in the supply of some energy by convection. It is important not to over-heat the plasma It should be examined frequently and stirred to insure uniform thawing When the mass has become a slush with only about 1/20 of the total remaining as ice, it is ready for centrifugation By close examination of the plasma, the fibrinogen can be seen as particulate material throughout the fluid

This material is then centrifuged in glass centrifuge tubes or chilled brass trunnion cups (International Equipment Co 2½" i d by 4½" deep) at room temperature at 1250 g for one minute The supernatant is poured off and a thin layer of fibrinogen can be seen lining the bottom of the cups This layer is allowed to remain, and with a new supply of plasma in the

whose optical density more closely approximates that of the unknown is used in this calculation. Finally, the fibrinogen content of the sample is multiplied by whatever factor is needed to express the result as mg. per 100 ml. of plasma. For example, if the O.D. of a 0.5 ml. sample is 0.500, and the O.D. of the 100 μgm. tyrosine standard is 0.550, then

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[2] Sawyer, W D, Alkjaessig, N, Fletcher, P, and Sherry, S A comparison of the fibrinolytic and fibrinogenolytic effects of plasminogen activators and proteolytic enzymes in plasma Thromb diath. haemorrh 5: 149, 1960

will just fit into the holes. This block is then placed in a freezing mixture of alcohol and dry ice. The small tubes are put in the holes and fibrinogen is pipetted into them. The solution freezes almost at once. After this process the tubes are placed in the deep freeze until they are thawed for use.

After the whole procedure is finished the containers and instruments used can be cleaned with a strong solution of sodium hydroxide. They can be set to soak in this mixture and on returning the next day they are easily cleaned Needless to say, all these instruments and containers should be scrupulously clean before use. The usual procedure is to use chromic and sulfuric acid cleaning solution on all apparatus This should be removed thoroughly with water and saline rinses before use.

Estimation of Clottable Protein in the Fibrinogen Preparation: One ml. of fibrinogen solution to be tested is mixed with 30 ml. of 0 85 per cent NaCl solution containing 1 ml. of M/5 phosphate buffer at pH 6.4, and 10.5 ml. of 1 per cent $CaCl_2$ The calcium is not precipitated by the phosphate. For convenience a 30 × 120 mm. tube of 50 ml capacity is used The $CaCl_2$ produces a less soluble fibrin and fibrin formed at acid pH is more easily rolled out than that formed in a more alkaline medium

To this diluted fibrinogen, 1 ml. of a 50 per cent glycerol solution containing about 100 Iowa units of purified thrombin is added "Thrombin Topical" may be used. Clotting occurs in a few moments, the opalescent fluid becoming cloudy. This mixture is allowed to stand for 30 minutes, either at room temperature or in the refrigerator

After the formation of fibrin the clot is rolled out with use of a stainless steel wire or rod, squeezing as much liquid from the clot as possible. To facilitate this latter step the clot is removed from the solution and rolled out on filter paper with gentle pressure. It is then washed twice with water and then twice with 0 85 per cent NaCl solution before being analyzed.

The clot may be dried and weighed It may be analyzed for nitrogen or tyrosine In the latter case the clot is placed in 0 5 ml. of 10 per cent NaOH for digestion The amount of tyrosine in the digested clot is then determined by the method of Folin-Ciocalteu To find the purity of the product the tyrosine equivalent of the solution is found for a 0.5 ml specimen taken before the clot is formed with thrombin A comparison of the two values then in hand, gives the percentage of the total protein that is clottable fibrinogen.

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same cups the centrifugation is repeated until all the fibrinogen has been "harvested."

It is emphasized that the preservation of low temperature is essential in all phases of this procedure. The brass trunnion cups or centrifuge tubes should be returned to the ice bath when not being handled and all objects used should be cool. If a cold room is available, that should be most nearly ideal.

Following this initial spinning, the fibrinogen is removed from the cups and deposited in a large metal beaker that has been placed in an ice bath An equal volume of ice cold saline is poured over the mass and the whole mixture is stirred by a hand stirrer. The device used is made by placing a No. 7 rubber stopper on the end of a strong glass rod. It is used as a stomper. Mechanical stirring denatures the fibrinogen. After thorough mixing, the material is returned to the cold centrifuge cups and centrifuged again at 1250 g for about 1 minute. After this centrifugation, the saline supernatant is discarded, the fibrinogen is removed and the mixing with saline is repeated. The amount of saline used each time is gradually decreased. The washing is repeated at least five times, and more times may be required before a final white fibrinogen is obtained. The last washing should be for three minutes, at the former speed to remove most of the saline.

The material is then suspended in about 200 to 300 ml. of saline and placed in a water bath at 35°C. The hand stirrer is again used to avoid denaturation and to insure complete heating of the mixture by keeping the liquid in constant motion until all the fibrinogen is dissolved and the final temperature is about 33°C

The solution is then placed in warm centrifuge tubes and centrifuged at room temperature for 2 hours at 1250 g Following this, the clear opalescent solution of fibrinogen is decanted into a graduate and the volume measured. A 2 or 3 ml. sample is taken for the analysis to be described.

The product itself is frozen with the aid of dry ice and alcohol and stored in the deep freeze for many months, or it can be freeze dried and stored in the powdered form for future use. When such frozen products are thawed they are placed in a hot water bath. If the hands can be immersed in the hot water comfortably, the fibrinogen container can be rotated until all ice is gone. Slow thawing without motion denatures purified fibrinogen.

Should the material be needed for analytical use at once, it is made up into a one per cent solution by dilution with 0.85 per cent NaCl solution. [illegible] should contain 10 per cent imidazole buffer by volume.

[illegible] 10 by 75 mm. and quick [illegible] make an aluminum block [illegible] 75 mm. serologic tubes

glycine-citrate-alcohol extraction was repeated and the twice-washed precipitate obtained. This was dissolved in a sodium chloride-sodium citrate solution (1/10 original plasma volume) in a H_2O bath at 37°C. Generally the fibrinogen goes into solution within one-half hour. This preparation is Blombäck's fraction I_o[1] and may be quick-frozen and stored in the deep freeze until ready to proceed with the tannic acid fractionation. Protein analysis was necessary at this stage and we used the biuret test of Robinson and Hogben.[3] If fraction I_o was frozen, it was thawed in a 37°C. water bath in about one-half hour. To the solution of fibrinogen, a 1 per cent tannic acid solution was added (0.08 ml. for each ml. of a 2 per cent protein solution). *The tannic acid was added dropwise with continuous* slow stirring at room temperature (26°C.). After standing 10 minutes, the preparation was centrifuged at room temperature in a Clay-Adams Table Top Laboratory Centrifuge at 1865 rpm (552 g) for 10 minutes. The supernatant solution was discarded. The precipitate (fibrinogen with traces of prothrombin) was dissolved at 37°C. in a solution of sodium citrate—polyvinylpyrrolidinone (300 ml used for each Gm. of protein present). The preparation was stirred slowly over a magnetic mixer and generally went into solution in about one-half hour. The redissolved fibrinogen was cooled down to 5°C. and 50 per cent ethyl alcohol was added to give a final concentration of 10 per cent alcohol The purified fibrinogen precipitated out and was collected following centrifugation at 2000 rpm (650 g) for 10 minutes at 10°C. This precipitate was redissolved in sodium chloride-sodium citrate to make a final volume that was 1/10 of the starting plasma volume. This purified fibrinogen was stable following either lyophilization or storage in the frozen state for at least 6 months.

Reagents

(1) 50 per cent ethyl alcohol.

(2) Glycine-citrate-alcohol solution, pH 6-7—1 M glycine, 4 per cent sodium citrate and 6½ per cent ethyl alcohol

(3) Sodium chloride-sodium citrate solution—0.45 Gm. NaCl + 2.0 Gm Na citrate in 100 ml water

(4) One per cent tannic acid

(5) Sodium citrate-polyvinylpryrrolidinone solution— 1 Gm. Na citrate + 1 Gm polyvinylpyrrolidinone in 100 ml saline.

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5. Purification of Fibrinogen

M. I. BARNHART and W. B. FORMAN

For our special purposes we wanted a fibrinogen product that was demonstrably free of the plasma proteins involved in coagulation. The Blombäck procedure,[1] utilizing ethanol fractionation of plasma and glycine extractions of impurities, provides good yield of high quality fibrinogen with AHF associated and only traces of profibrinolysin and prothrombin However, we needed fibrinogen that was immunologically free of these substances. As a result of conversation with Casillas, we tried the method developed by him with Pavlovsky and Simonetti[2] for separating AHF from fibrinogen. This method employs Blombäck's fraction I_o as the starting material and utilizes tannic acid to selectively separate out fibrinogen from AHF.

In our experience with this modified method on human, canine and bovine plasmas, fibrinogen was obtained which was free of AHF and did not contain profibrinolysin or prothrombin when tested with immunologic methods. A single precipitin band (in the Beta region) was formed against specific anti-fibrinogen sera and anti-human plasma during immunoelectrophoresis Concentrations up to 10 mg./ml. were checked and the fibrinogen appeared to be homogeneous by this method of testing. In diffusion test with agar gel using modified Ouchterlony plates, no profibrinolysin or prothrombin was found This purified fibrinogen was generally around 97 per cent clottable and the yield from fraction I_o was 35-40 per cent Such fibrinogen was stable in the frozen state and the lyophilized preparation containing sodium chloride and citrate redissolved readily on addition of water.

Procedure. Details of the method for fibrinogen purification follow. Composition of the reagents is given at the end Citrated fresh or outdated plasma was chilled to 2°C. in a salt-ice bath To this was added slowly cold 50 per cent ethyl alcohol to produce a final concentration of 8 per cent alcohol. The mixture was slowly stirred, using a magnetic mixer during the addition, and then stood for one-half hour The precipitate (fraction I) was centrifuged down in plastic tubes in a Servall refrigerated centrifuge at 2000 rpm (650 g) for 10 minutes The supernatant solution was discarded and the rather sticky precipitate was mixed with a glycine-citrate-alcohol solution. For each 100 ml of plasma processed, 20 ml. of glycine-citrate-alcohol was added to the precipitate, transferred to a cold beaker, and mixed slowly at −2° to 0°C. for one-half hour. This extracts a major portion of the impurities carried down with the fibrinogen by fractionation in 8 per cent alcohol. The undissolved precipitate was centrifuged at 2000 r.p m. (650 g) for 10 minutes and again the supernatant discarded. The

press: Fermpress-Apparatus, H 207W (J. Mueller, Zurich, Switzerland, or Wheaton). (13) Virtis Freeze Dry apparatus or equivalent and cylinder of dry N_2 gas (14) Six hundred ml. polypropylene beakers.

Procedure:

1. *Preparation of Plasma* Normal blood is drawn by venepuncture and collected directly into an evacuated siliconized blood donor bottle containing 0 02 volume of 19 per cent sodium citrate for each volume of blood. If a siliconized bottle is not available, one may be prepared from a Baxter ACD container by withdrawing with a syringe and 19 gauge needle its content of ACD solution, rinsing twice with 100 ml. of saline by injecting this volume into the bottle and withdrawing the rinses. The proper volume of 19 per cent sodium citrate then is injected into the bottle and the bottle evacuated. One single, *careful* puncture of the rubber stopper through the "inlet +" point is made for the entire operation, during which the stainless steel syringe needle must remain in the stopper A multiple-punctured stopper frequently will not hold vacuum.

The citrated blood is centrifuged for 20 minutes in 50 ml. plastic tubes at 2900 rpm (1800 g) at 4°C, and the plasma is carefully aspirated to collect 95 per cent of the total plasma volume without disturbing the buffy coat. Platelet-poor plasma is prepared by recentrifugation for 1 hour at 1800 g at 4°C.; 90 per cent of this plasma volume is aspirated with a 50 ml. syringe and needle (16 gauge blunt end) The plasma is processed immediately, or shell-frozen in dry ice-acetone mixture and stored at −15°C until used. Before use, frozen plasma is melted quickly at 38°C in a water bath but warmed only to a temperature of 20°C Fresh ACD (acid citrate dextrose) plasma or ACD plasma from bank blood may be used, the latter, obviously, only if it is still in good condition.

2. *Adsorption of prothrombin* 20 mM (5 Gm per liter) of crystalline magnesium sulfate are dissolved slowly in the plasma with the aid of gentle mechanical stirring (15 minutes) at a constant temperature in a 20°C water bath $BaSO_4$, 90 Gm /liter, next is added slowly to the plasma as a dry powder and the suspension is stirred for 1 hour The bulk of the $BaSO_4$ is removed by centrifugation at 20°C for 15 minutes at 2800 rpm (1800 g). The partially deprothrombinized supernatant is treated with another 90 Gm /liter charge of $BaSO_4$ in the same manner as the first The prothrombin time of the supernatant plasma should exceed 45 minutes, if it does not, a third treatment with 45 Gm. $BaSO_4$ per liter may be necessary The small amount of $BaSO_4$, usually carried over in the supernatant, is removed by centrifugation at 16,000 g (Servall or Spinco) for 20-30 minutes. For volumes in excess of 500 ml, clarification in the Sharples Supercentrifuge at 38,000 r.p m. and a flow-rate of 20 ml per minute is convenient.

6. Preparation of Fibrinogen by Glycine Precipitation (Method of Kazal, Miller, Amsel and Tocantins[1])*

L. A. KAZAL, G. F. GRANNIS and L. M. TOCANTINS

Object To prepare fibrinogen from small or large amounts of plasma without the use of low temperature, organic solvents or high concentrations of neutral inorganic salts or from Cohn Cold Ethanol Fraction I.

Principle. The method is based on the direct salting-out of fibrinogen from plasma by an amino acid, glycine [1] Fibrinogen, as the least soluble of the plasma proteins, is the first to separate from plasma by precipitation technics.[2] This same event occurs when glycine is dissolved in plasma, in suitable concentration, with the important difference that whereas most agents will precipitate other proteins of plasma as the concentration of the precipitating agent is increased toward saturation, glycine at saturation will precipitate only fibrinogen and a beta globulin, leaving the other proteins in solution (salting-in action). By selecting a temperature of 20°C. and a concentration of glycine corresponding to 0.78 saturation (2.08 M), only fibrinogen precipitates

Preparation of Fibrinogen from Plasma

Reagents (1) ACD or 19 per cent sodium citrate plasma (see table 1, p 000) (2) 0 055 M sodium citrate, Merck Reagent $Na_3C_6O_7 \cdot 2H_2O$, pH 7.4. (3) $BaSO_4$, Baker Analytical Reagent Powder (4) Glycine (NH_3-free). (5) Magnesium sulfate·$7H_2O$, cryst , reagent

Apparatus (1) Siliconized 50 ml glass syringe, 19 gauge regular and 16 gauge, 4" blunt-end stainless steel needles (2) Silicone-coated Baxter TransfusoVac Bottle with ACD solution (Baxter Laboratories, Morton Grove, Ill.) (3) International PR-2 Centrifuge or equivalent for 1800 g. (4) fifty ml and 100 ml plastic centrifuge tubes (5) Dry ice acetone bath (6) Temperature-controlled water bath at 20°C (7) Mechanical stirrer. (8) Beakers or precipitating jars (9) Sharples Super-Centrifuge, Type T-1P, Clarifier bowl or Spinco Model L ultracentrifuge with No 21 rotor, or equivalent to give approximately 56,000 g (10) Servall SS-1 Centrifuge, 32,500 g, (50 ml tubes) (11) Wheaton No-Sol-Vit Flint serum vials, S-104E and S-205 red rubber stoppers, S-46-87, single tear off aluminum seal #20-10 TL (Wheaton Co , Millville, N J). (12) Aluminum-seal hand

* This investigation was supported by Grant No H-3544 of the National Heart Institute, N I H , U S P /H S

$BaSO_4$ adsorption is best achieved at 20°C or at room temperature; it is less effective at 0°C. Fibrinogen prepared from poorly adsorbed plasma is subject to spontaneous clotting and the presence of prothrombin in such fibrinogen preparations is readily demonstrated by recalcification in the presence of thromboplastin

2. *Temperature*. The procedure is designed for 20°C in order to control both the solubility of glycine and the precipitation of protein contaminants. The solubility of glycine increases measurably with temperature (1 89 M glycine at 0°C.; 2.68 M at 20°C in distilled water). Since fibrinogen free of gross protein contaminants is precipitated in 0 78 saturated glycine (2 08 M at 20°C.) solution, temperature control is necessary to maintain this molarity Furthermore, above 2 08 M, conditions favor the co-precipitation of a beta globulin contaminant. Under these conditions about 5 per cent of the total fibrinogen of plasma is not precipitated, but the isolation procedure is simplified

3 *Centrifugations* The successful preparation of very highly clottable protein is dependent also upon the efficient removal of any plasma proteins which remain in that part of the glycine supernatant trapped in the centrifuged fibrinogen precipitates These may be removed by washing the precipitate with 2 08 M glycine at 20°C, or preferably, as in the described procedure, by centrifugation at high speed with the Sharples centrifuge which produces a thin paste of precipitate essentially free of contaminating plasma supernatant Two precipitations under these conditions provide 98 per cent clottable protein Other centrifuges may be substituted for the Sharples centrifuge, as specified, provided the supernatant contamination problem is borne in mind In order to control this problem, gross contamination may be followed with paper electrophoresis by standard technics; trace contamination is best detected by careful application of relatively larger volumes (0 03 ml) of concentrated solution to the paper

4 *Glycine* The fibrinogen prepared by this procedure contains a trace of glycine, 0 015 M, in 0 25 per cent fibrinogen This concentration of glycine is without effect on the thrombin-fibrinogen reaction[3] If it is desired to remove glycine, dialysis at low temperature against 0 0125 M or 0 05 M sodium citrate buffer at pH 6 3 or 7 4 may be employed, several days are required for complete removal Some of the fibrinogen precipitates at refrigerator temperatures but dissolves on warming to 38°C The trace of glycine also may be removed by passage through a column of Sephadex G 25 equilibrated with 0.055 M sodium citrate

5 *Analytical Procedures* Total nitrogen may be obtained by micro-Kjeldahl analysis using a factor of 6 0 for conversion of protein Since the fibrinogen solution contains some glycine, dialysis against citrate buffer is necessary if total nitrogen is to be determined The same precaution must be

3. *Precipitation of crude fibrinogen by glycine.* To each liter of $BaSO_4$ supernatant at 20°C., 165 Gm of glycine are added in small aliquot portions. The solution is stirred slowly for 30 minutes, avoiding the formation of foam as much as possible The precipitated fibrinogen is separated by centrifugation as in step 2. The supernatant may be saved, dialyzed free of glycine and used for the preparation of other proteins.

4. *Purification* The fibrinogen paste is dissolved in a volume of 0.055 M sodium citrate at pH 7.4 equal to the volume of the first $BaSO_4$ supernatant. A glass rod is used to disperse the gelatinous paste into smaller pieces; solution is completed by mechanical stirring for 30 to 60 minutes After clarification by centrifugation at 1800 g, 165 Gm. of glycine are added, as before, for each liter of solution and the solution stirred for 30 minutes The precipitated fibrinogen is collected as in step 2 and the supernatant is discarded. (If a high speed centrifuge is not available, step 3 should be repeated again to insure the removal of the traces of supernatant plasma protein trapped in the centrifuged precipitate)

5. *Clarification and lyophilization* The fibrinogen precipitate is dissolved in a volume of 0 055 M sodium citrate buffer at pH 7.4 equal to ¼ or ⅓ volume of the first $BaSO_4$ supernatant. The solution usually is clear; if particulate matter is present, clarification by centrifugation for 30 minutes in the Servall or Spinco Model L at 32,700 g is essential The clear solution is filled into serum vials, plug-frozen in acetone-dry ice mixture, and freeze-dried for 48 hours in a vacuum chamber A vacuum oven modified to permit it to be connected by large bore rubber tubing to the freeze-drying apparatus is very convenient The vacuum then is dispelled with nitrogen gas, the containers capped with rubber stoppers in an N_2 atmosphere, and sealed with pressed-on aluminum seals All preparations are stored at −15° C until used. (Air may be used instead of nitrogen)

Comments and Precautions Fibrinogen with a high order of clottable protein may be obtained by this method from either fresh or frozen plasma, provided certain precautions are observed

1 *Adsorption.* The adsorption of prothrombin by $BaSO_4$ from citrate-plasma is necessary to prevent subsequent spontaneous clotting of fibrinogen solutions $BaSO_4$ does not adsorb prothrombin in the presence of citrate ions. Magnesium ions ($MgSO_4$) added to plasma reduce the desorbing activity of the citrate anion by forming a non-ionized complex with citrate, thereby providing suitable conditions for adsorption A sufficient concentration of free citrate ion remains to retard coagulation of plasma. $BaSO_4$ adsorption must be started immediately after the $MgSO_4$ salt is dissolved, and plasma containing $MgSO_4$ should not be stored for any length of time, in order to avoid spontaneous clotting

Merck reagent grade sodium citrate dihydrate is dissolved in water and diluted to 1 liter. (3) Stock 3.75 M sodium chloride, 219.4 Gm. of Merck reagent grade sodium chloride is dissolved and diluted in water to 1 liter. (4) Dilute sodium citrate (0 055 M), 50 ml. of 1.1 M sodium citrate is diluted with water to 1 liter. (5) Dilute citrate-saline solution (0 022 M sodium citrate—0 15 sodium chloride), 20 ml 1 1 M sodium citrate and 40 ml. of 3.75 M sodium chloride are diluted with water to 1 liter. (6) $BaSO_4$, Baker Analytical Reagent Grade Powder (7) Glycine, (NH_3-free), 99 5 *per cent minimum* assay. (8) Sephadex G-25, *Pharmacia*, coarse grade

Apparatus· (1) Stainless steel pots, 5 gal, or 20 liter Pyrex jars. (2) Large temperature-controlled water bath. (3) Two 2″x48″ glass chromatographic columns. (4) Other equipment (centrifuges, stirrers, etc.) same as for plasma procedure.

Procedure·

1. *Removal of residual prothrombin.* Fresh Cohn Fraction 1 is minced with scissors and dissolved in dilute citrate-saline, using one liter of solution for each 150 Gm. (wet weight) of Fraction 1, in a 5 gal. stainless steel pot maintained at 20°C. in a controlled temperature bath. The solution contains 2-3 per cent protein (calculated as fibrinogen) of which approximately 70 per cent is clottable with thrombin In order to minimize deterioration of product during subsequent purification procedures, the solution is treated with $BaSO_4$ to adsorb residual prothrombin. 5 Gm of $MgSO_4 \cdot 7H_2O$, followed by 90 Gm of $BaSO_4$ powder is added for each liter of solution The mixture is stirred for one hour and then centrifuged, first in the PR-2 International Centrifuge (2000 rpm, 10 min.) and then in the Sharples Centrifuge at 38,000 rpm (52,600 g) Additional treatments with full or half-charges of $BaSO_4$ may be necessary to remove prothrombin, as in the procedure for plasma. When the solution has no detectable prothrombin activity, it is shell-frozen in large bottles and stored at −15°C for future work-up †

2 *First and second glycine precipitations* The prothrombin-free preparation was thawed rapidly and adjusted to 20°C For each liter of solution, 165 Gm (2 2 moles)of solid glycine are added slowly with stirring to precipitate fibrinogen After 60 minutes the bulky precipitate is collected by centrifugation (PR-2 Centrifuge, 2000 rpm, 20 minutes) and dissolved in one liter of dilute citrate solution (0 055 M) for each liter of starting

† Storage at this stage is required because of the large volumes (e g 12 liters) of solution being processed Storage for 2 days before precipitation of fibrinogen has been satisfactory according to current experience, however, a preparation held in storage for 6 months at this stage of preparation yielded an inferior product

exercised with regard to the use of the biuret reagent for the determination of protein, because glycine develops a color with this reagent. A very reliable method for the determination of fibrinogen concentration is by absorbance at 282 mμ according to the technic of Blombäck and Blombäck;[2] methods for the determination of clottable protein also are described. Other methods for clottable protein obviously are applicable provided the precaution regarding glycine is recognized, when necessary.

Results: In a typical experiment fibrinogen was obtained from 1.75 liters of citrate plasma in a yield of 60 per cent of that present in the $BaSO_4$-adsorbed plasma. Before lyophilization, 97.8 per cent of the protein in the preparation was clottable; after lyophilization, 94 3 per cent. The fibrinogen content of the powder was 20.2 per cent of total solids; the nitrogen content, 16 5 per cent; the extinction coefficient ($E^{1\%}_{282\,m\mu}$) in 0.055 M citrate buffer, at pH 7.4, 14.4

Paper electrophoresis of glycine-precipitated fibrinogen showed one component. By Tiselius moving boundary electrophoresis,* 98 per cent of the lyophilized protein was present as a single, slightly skewed component (phosphate-NaCl buffer, pH 7.4, ionic strength 0 12 at 20°C.). Two components in ratio of 85 per cent to 15 per cent were observed by analytical ultracentrifugation.†

The lyophilized fibrinogen powder which contains sodium citrate was a white, amorphous solid, readily soluble in water. The dried powder has good stability on storage: in our present experience, in excess of 2 years at −15°C. under nitrogen

Solutions of glycine-precipitated fibrinogen (0 25 per cent) are clotted by 10 units of thrombin in 8 seconds in the presence of a trace of calcium chloride (0 003 M), but somewhat slower (38 seconds) in the absence of calcium Prothrombin and plasmin are not present, but a trace of plasminogen and fibrin stabilizing factor have been found. The concentration of antihemophilic globulin (factor VIII) is estimated at 4 per cent of normal plasma. Where plasminogen is not a factor, glycine-precipitated fibrinogen obtained by the described procedure has served admirably as a reagent in clotting assays

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Reagents. (1) Fresh Cohn Fraction I of human plasma obtained as a wet, non-frozen paste ‡ (2) Stock 1 1 M sodium citrate—323.5 Gm of

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of Cohn Fraction 1 (equivalent to approximately 45 liters of plasma) have been processed as a unit operation.

The fibrinogen prepared by this procedure has the same purity and the same kind of trace contaminants as that obtained from plasma, except that it is glycine-free.

It has served satisfactorily in many assays requiring fibrinogen, except where plasminogen-free reagent is required Preparations have been stable for a period of 2½ years without any evidence of loss of clottability or solubility.

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7. Assays for the Fibrin Stabilizing Factor (FSF)

L. LORAND†

I. *Background Information*

As its name‡ implies, the biological function of the "fibrin stabilizing factor" (FSF) is to stabilize the clot structure This is effected through a cross-linking process. Clots formed in the absence of FSF are mechanically weaker and dissolve in a number of solvents in which normal plasma clots remain stable This added strength of the clot, brought about by the action of FSF, appears to have important physiologic consequences Stabilized clots resist lytic enzymes better, a circumstance which hitherto has not been sufficiently considered in connection with thrombolytic therapy Clot retrac-

† Investigations pertinent to this work were aided by a U S Public Health Service Research Career Program Award (HE-K6-3512) and by a grant (H-2212) from the National Heart Institute, National Institutes of Health, United States Public Health Service

‡ Recently designated factor XIII by the International Committee on Nomenclature of Blood Clotting Factors

solution. To this solution, which contains approximately 2 per cent protein and is 90 per cent clottable by thrombin, is added 165 Gm. of solid glycine, and fibrinogen is precipitated as before. After 1 hour, the solution is passed through the Sharples Centrifuge, the tough paste of fibrinogen is recovered and minced into 1 liter of dilute citrate solution (0.055M) for each liter of starting solution, and stirred slowly overnight at 4°C. The solution is then warmed to 37°C. to dissolve precipitated protein and a small amount of insoluble material is removed by centrifugation (PR-2, 2400 rpm, 10 minutes). The fibrinogen solution contains approximately 1.7 per cent protein and is 95-98 per cent clottable.

3. *Removal of residual glycine by gel filtration*· Two 2" diameter chromatography columns are filled to a bed depth of 32" with Sephadex G-25, coarse grade, which had been suspended repeatedly in distilled water to remove "fines" and subsequently equilibrated with 0.055 M sodium citrate. Two liters of sodium citrate solution are passed through each column prior to use. The fibrinogen solution, diluted to 1.2 per cent protein, is applied to the column until fibrinogen appears in the eluate (a volume of 550 ml.), after which 1400 ml. of 0.055 M sodium citrate is passed through the column. The fibrinogen fraction is easily detected visually by its opalescence. Each column is operated continuously: following the passage of sodium citrate, another 550 ml. of fibrinogen solution is introduced, followed by 1400 ml of sodium citrate In this way, 2 to 3 liters of fibrinogen solution can be processed on each column in a day. Initially, 25 ml fractions are collected and analyzed for absorbance at 282 mμ[1] (fibrinogen fractions) and for α-amino nitrogen[4] (fractions after fibrinogen) In subsequent runs, since the volumes at which fibrinogen and amino acid appear are reproducible, the major portions of the fractions are collected in bulk and only the leading and tailing portions are collected for analysis of amino acid. All but the leading and tailing fractions of fibrinogen are pooled, diluted to a concentration of 1.2 per cent fibrinogen and portioned into small bottles for lyophilization The aliquot volumes are plug-frozen immediately and stored at −10°C., then lyophilized The lyophilized samples are filled with nitrogen, capped and stored at −10°C

Comments. Fibrinogen prepared from Cohn Fraction I by the above procedure is similar in its properties to that obtained from plasma The use of Cohn Fraction I has the tremendous advantage of high yield, since fibrinogen is concentrated in this fraction. The processing of the fibrinogen solution at approximately 3 per cent concentration, instead of at 0 3 per cent as in the plasma procedure, obviously offers an advantage in terms of the relatively smaller amount of glycine required for precipitation of equivalent amounts of fibrinogen As much as 600 Gm (wet weight)

to its insoluble core within 2 hours with only occasional shaking Results should, however, also be checked about 16 hours later.†

When using 30 per cent urea as a dispersing agent, the same clot is prepared in the form of a film of about 2 mm. thickness, and dissolving in urea is carried out by mechanical shaking at 37°C lasting 16 to 24 hours

C. *Estimation of the cross-linked cores of clot structures.* The monochloroacetic acid-insoluble portion of a clot is believed to represent the covalently cross-linked core brought about by the action of FSF. It could be noted either by qualitative indexing (e g , + to 4+) or, preferably, by quantitatively estimating the monochloroacetic acid-insoluble protein residue which remains after 16 hours in the acid The residual clot is centrifuged, and the acid is decanted. The protein sediment is then washed and centrifuged repeatedly (*ca* 5 times) in 10 ml. lots of 0.9 per cent NaCl The final residue is hydrolyzed in boiling alkali and its protein content determined by Folin's phenol reagent (Ztschr phys. Chem. *304* 53, 1956). Values are expressed in terms of Armour crystalline, dry (105°C. for 2 hours) bovine serum albumin as standard.

D. *Relation of Cysteine to FSF activity Choice of fibrinogen for the assay.* From what has been presented, it is clear that a relatively simple test can decide whether the blood clot of an individual (formed by intrinsic fibrinogen, FSF, and thrombin) belongs to the stabilized variety or not All that is required is to mix the clot with an equal volume of 2 per cent monochloroacetic acid or 60 per cent urea and note whether dispersion occurs Such a test, though useful as it is, can only inform us in a crude way about the absence or presence of FSF activity in blood It is a considerably more complex problem to quantitate FSF activity (in plasma or in purified fractions) on extraneous fibrinogen preparations ‡ We found that FSF requires the addition of cystine to show full potency,§ and, when cysteine is incorporated in the clotting mixture, complications arise from the fact that fibrinogen preparations are contaminated‖ by some form of FSF which apparently turns into the active principle when cysteine and thrombin are added. One thus faces the dilemma of either not measuring full FSF potency in the absence of cysteine, or showing variable and anomalously high activities for the factor in the presence of cysteine, depending on what kind of fibrinogen substrate one uses.

† Actually, twice this amount of fibrinogen (10 mg) in twice the volume (5 ml) is still manageable in the same form Naturally, 5 ml of 2 per cent monochloroacetic acid will be used for solvent

‡ Bovine fibrinogen reacts with human FSF and vice versa to produce stabilized clots

§ Cysteine, however, does not replace FSF

‖ "Clottability" determinations are totally inadequate to detect these contaminations

tion might also be influenced. A deficient clot structure, formed in the absence of FSF, apparently does not support the growth of fibroblasts as well as a normal plasma clot does; hence, secondary hemorrhaging and poor wound healing may result.

Although originally discovered in serum, FSF is now primarily regarded to be a plasma protein with only traces of activity remaining in serum FSF activity has also been recognized in platelets. In the phylogenetic scale, FSF activity has thus far been demonstrated in human, bovine, canine, rabbit, fish, and cyclostome blood plasmas.

According to our present knowledge, the actual clotting phase in blood can be analyzed as follows.

Fibrinogen, FSF, and thrombin (in the presence of calcium ions) are the initial reactants, forming fibrin monomer and activated FSF, the latter abbreviated as FSF*. The fibrin monomers, in the absence of FSF*, could undergo only reversible aggregation to yield a clot which is soluble in urea or acid. In the presence of FSF*, however, cross-links develop which endow the clot (plasma clot or stabilized clot) with greater mechanical strength and stability in the solvents mentioned.

II. *Bioassays for FSF Activity: The Clot Solubility Tests*

A. *Solvents* During the past decade, we have developed a number of bioassay procedures (see II E) whereby the activity of FSF in blood and in purified blood fractions may be measured. These tests are based on differences in solubilities between a clot which is stabilized by the action of FSF and one which is not Thus, they are intended to recognize deficiencies in the clot structure One per cent monochloroacetic acid† or 30 per cent urea are the most widely used selective solvents; our recent assays utilize the former, mainly on account of its rapid clot dispersing effect.

B *Size of the clot.* In the solubility tests, the solvent has to penetrate the gel from without, thus, consideration must be given to the dimensions of the clot itself Ideally, the influence of several factors (such as concentration of proteins present, nature and concentration of the dispersing agent used, mechanical agitation and temperature) should be analyzed From experience, however, we can say with certainty that a clot plug can be prepared from 5 mg of fibrinogen in a volume of 2 5 ml. in a test tube of 18 mm. in diameter, so that the admixing of 2 5 ml of 2 per cent monochloroacetic acid will reduce such a clot at room temperature (20–25°C.)

† Concentrations refer to final concentrations of the compounds in the assay systems Clot solubility tests could also be based on other solvents, such as 1 M NaBr at pH 5 4, 1 per cent neutral "Alkonox" detergent, or some weak acids and alkalies at specified temperatures

FSF, and thrombin prior to adding the dispersing agent to the clot.† Such longer incubation results in a shift of the critical FSF concentration to lower values, reaching a fixed "threshold" after 4 hours, and apparently not changing thereafter.‡

The "apparent threshold" existing for FSF under specified conditions was used to detect individuals with lowered FSF activities (i e., higher "apparent threshold"). In a screening of more than 100 persons, about 90 per cent of the individuals tested showed a sharp distribution of "FSF thresholds," in 30-minute reactions, centering between 0.1 and 0 2 ml. of plasma, whereas approximately 10 per cent gave values of 0 6 ml or higher.

Though one of the simplest of FSF activity tests, in the light of our present knowledge, the "threshold method" is hard to analyze. Even ignoring the FSF-related potential which contaminates most fibrinogen preparations, there are several simultaneous reactions (at least two of them competing for thrombin), discussed under II D, when fibrinogen and FSF are mixed with thrombin It is not known what fortuitous combination of these might be responsible for a given "threshold" value.

b *The "apparent optimal ratio" method* In another type of one-stage method, the concentration of FSF is kept constant and that of fibrinogen is varied in the clot mixture (see figure 76 on p 242 of ref 1) Under these conditions, an "apparent critical ratio" between the amounts of fibrinogen and FSF is indicated for the production of stabilized clots. It must be pointed out, however, that in the light of newer understanding, discussed under II D, this kind of a test is again hard to interpret An apparent optimal ratio could conceivably arise from the fact that both fibrinogen and FSF are competing for thrombin during the half-hour incubation period If the relative concentration of fibrinogen over that of FSF is excessively high, the activation of FSF to FSF*, and thus the stabilization of the clot, could be delayed.

2 The two-stage method (see figures 80 and 81 on p 253 of ref 1, and also ref 2).

Because of the circumstances discussed (II D and E 1), one-stage methods have great limitations in measuring FSF activity. Only the use of a two-stage assay can provide a good appraisal FSF has to be incubated first with thrombin to provide activation of FSF* and then, in the second

† If 20 mM cysteine is incorporated in the clotting mixture, the complications discussed under step 3 of II D must be kept in mind

‡ It is conceivable, however, that removal of O_2 will affect this behavior

§ Thrombin from the activation medium should possibly be quenched by adding one of the thrombin inhibitors (such as 20 mM N_α-toluene-sulfonyl-L-arginine methylester) 1 minute before the admixing of the fibrin solution

Consider producing a clot by mixing solutions of fibrinogen, FSF, cysteine,† calcium chloride, and thrombin A number of reactions will take place with a variety of time courses.

1. Fibrinogen is converted to fibrin through the splitting off of fibrinopeptide by thrombin.

2 The added FSF is activated by thrombin to FSF* in a transient manner, since FSF* has quite a short life in air.

3. Cysteine and thrombin activate the FSF-related compound, which is intrinsically present in fibrinogen preparations, to FSF*.

4. Fibrin and FSF* interact to produce a cross-linked network which is finally measured by the solubility test.

Step 3 should obviously be eliminated as much as possible by using a fibrinogen preparation which is refractory to cysteine. Such fibrinogens are, however, hard to obtain; inherent FSF-related impurities could either be inactivated or removed. Partial destruction may be achieved by moderate heat treatment.‡ Removal could be achieved so far only on a small scale by a stepwise elution chromatography using diethylaminoethylcellulose column (see figures 77 and 78 on pp. 243 and 244 of ref. 1).

E. *Some FSF activity tests.*

1 One-stage procedures; based on the simultaneous interactions of fibrinogen, FSF, and thrombin.

a. *The "apparent threshold" method* Our first test to quantitate FSF activity made use of the finding that there is an "apparent threshold" amount of FSF which is required to produce a minimum visible piece of urea- or monochloroacetic acid-insoluble clot from a given amount of fibrinogen (see figure 75 on p 241 of ref 1) In such tests, the concentration of FSF is being varied, whereas those of fibrinogen and thrombin, as well as the time of interaction between the three, are kept constant. It is possible, of course, to lengthen the time of reaction between fibrinogen,

† Usually 20 mM neutral fresh cysteine is included in these test mixtures. If the clot is allowed to develop over a period longer than a half an hour, oxidation of the thiol may be prevented by nitrogen atmosphere, or by the use of 20 mM glucose and trace amounts of glucose oxidase (Sigma), or by a layer of floating mineral oil

‡ A solution of 1 per cent fibrinogen (about 95 per cent clottable) in a *pH* 7 5, μ 0 18 Tris-HCl buffer is kept at 40° C for 3 hours and frozen overnight Warming and freezing may be repeated (if clottability does not diminish) on successive days until the preparation obtained will yield monochloroacetic acid-soluble clots after 12 to 16 hours of incubation with 0 2 mM $CaCl_2$, 20 mM cysteine, and 1 unit of throm-

[illegible]

precipitation of fibrinogen with ammonium sulfate

15 minutes for the gel to develop and for FSF* to effect cross-linking Then add 2.5 ml. of 2 per cent monochloroacetic acid and estimate the acid-insoluble residue as described in II C above.

One unit of FSF could be defined as the activity which produces 2 mg of acid-insoluble clot residue under the conditions given.

References

[1] Lorand, L.: Properties and significance of the fibrin stabilizing factor (FSF). Thromb diath. haemorrh. *3* (suppl. 1). 238, 1962.

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step, FSF* will be mixed† with purified fibrin and the extent of cross-linking is measured by the amount of acid-insoluble gel that is produced during a given period.

Purification of fibrin, used in the second stage of the assay, is carried out according to the method of Lorand and Middlebrook (Biochem. J. 52: 196, 1952). A fibrinogen solution (about 0.5 per cent in protein at pH 7.5 and μ: 0.18 in Tris-HCl-NaCl buffer with NaCl contributing 0.1 M) is mixed with dilute thrombin so that clotting is initiated in about 2 minutes The clot is collected as it forms by means of a glass rod over a period of 3 hours and it is then dissolved with the aid of sufficient volume of 40 per cent urea solution to yield a final concentration of 30 per cent in urea. Fibrin is reprecipitated again by a 60-fold dilution of the protein solution with a pH 6.5 phosphate buffer (containing 0.09 M KCl, 0.015 M Na_2HPO_4 and 0.03 M KH_2PO_4). The precipitate is collected and redissolved in urea as before, and the procedure is repeated 3 more times. The final fibrin precipitate is dissolved into sufficient 2M NaBr solution (pH 5.4 with acetate buffer) to yield a solution of fibrin with about 1.5 per cent protein and 1 M NaBr concentration (Donnelly et al.: Arch biochem. biophys 56 369, 1955). Three-tenths ml. of such a fibrin solution is added in the second stage of the FSF assay to the 2.2 ml. activation medium.‡ Gelation is thus induced by diminishing the concentration of NaBr to about 0.12 M.

The two-stage method allows a meaningful definition of unit activity for FSF. There are a number of variables (e g, time, thrombin concentration, etc.) on which definition of activity could be based We are still in the process of refining this test, but the following simplified specifications may now be recommended as a working outline.

*First stage or activation of FSF to FSF**. At 25°C. mix 0 5 ml of fresh neutral 0.1 M cysteine dissolved in a pH 7.5, μ. 0.18 Tris-HCl-NaCl (NaCl contributing 0 1 M) buffer, 0 5 ml of 1 mM aqueous $CaCl_2$ and 5 units of thrombin dissolved in the above buffer. Make up total volume to 2 ml with the buffer, then add 0.2 ml of the FSF-containing solution in various dilutions with Tris buffer Incubate for 10 minutes.

*Second stage or cross linking of fibrin with FSF**. At the end of 10 minutes' incubation period, add 0 3 ml of the purified fibrin solution in 1 M NaBr (containing 4 mg. of protein) to the activation medium Allow

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† See footnote §, page 243

‡ Fibrin purified as such does not produce acid-insoluble clots if incubated, in the absence of FSF*, in 20 mM cysteine and 0 2 mM $CaCl_2$ over a period of 24 hours. The FSF-related impurities, at least those sensitive to reactivation by cysteine, are thus greatly destroyed or removed during purification. The fibrin solution is stored at 4° C.

fibrin plate but not on the heated fibrin plate. It is not possible to use these tests as presently constituted to demonstrate that only fibrinolysin is present in the test sample or the absolute ratio of fibrinolysin to activator. However, if demonstrated by other means that only fibrinolysin is present in the test sample, its concentration can be estimated through measurement of the size of the lysed area.

Reagents and Apparatus: Fibrinogen. A source of relatively pure fibrinogen is required. Astrup and Mullertz recommend fibrinogen prepared from oxalated ox blood which is treated with tricalcium phosphate and the fibrinogen precipitated with ammonium sulfate.[1] The fibrinogen prepared by the free-thaw technic of Ware et al.[3] may also be used The preparation should be buffered at pH 7.8 with diethyl barbiturate buffer and the ionic strength of the solution used to form the fibrin film should be 0 15. The concentration of the fibrinogen in the solution to form the fibrin can be 0.1 per cent or 0 2 per cent, the higher concentration of fibrinogen produces the more stable fibrin film

Thrombin A source of relatively pure thrombin is required Astrup and Mullertz[1] in their original experiments used thrombin obtained from Løven kemiske Fabrick, Copenhagen Thrombin Topical, Parke, Davis & Co, Detroit, is also satisfactory The powder is dissolved in a 0 9 per cent NaCl solution to give a preparation with 100 N.I.H units/ml

Buffer Astrup and Mullertz[1] recommend the use of diethyl barbiturate buffer prepared by adding 662 ml of 0 1 M sodium diethyl barbiturate to 338 ml. of 0 1 M HCl and diluting with 320 ml. water. The pH should be 7 8 and the ionic strength 0 05 The ionic strength of the solution used to form the fibrin must be low and constant, and to insure low ionic strength, fibrinogen solutions of relatively high ionic strength must be diluted with the low ionic strength buffer to bring the final ionic strength to about 0.15.

Petri dishes Dishes of 10 cm diameter are used Bottom and cover should be even so that they can be stacked in an incubator The dishes are heat-sterilized before use in the test

Procedure Nine ml of the diluted fibrinogen solution (0 1 or 0 2 per cent, pH 7 8, μ = 0 15) *is pipetted into each horizontal petri dish and* clotted with 0 2 ml of thrombin solution Unheated fibrin plates are ready for the addition of the test solution as soon as clotting has occurred Petri dishes containing the fibrin clot which is to be heated are placed in an oven which has been preheated to 80°C The dishes are allowed to remain in the oven at 80°C for 30 minutes They should cool to 37°C. or less before the addition of the test solution

Solutions to be tested (0 030 ml) are added in a small drop to the surface of the fibrin in the petri dishes from a 0.1 ml pipette, graduated in 0 001 ml Three separate drops can be placed on each plate. The plates ubated for 18 to 20 hours at 37°C.

CHAPTER X

FIBRINOLYSIN PRECURSORS AND INHIBITORS

1. *Estimation of Fibrinolytic Activity of Plasma or Serum: Fibrin Plate Method, Unheated and Heated (Methods of Astrup and Mullertz[1] and Lassen[2])*

Adapted by M. M. GUEST

Object. The tests are designed to semi-quantitate the activated proteolytic enzyme of plasma, fibrinolysin (plasmin), and to determine whether or not the activity results partly or entirely from the presence of an activator of profibrinolysin (plasminogen)

Principle Fibrin is one of the physiologic substrates which fibrinolysin proteolyzes The amount of fibrin solubilized in a given time is dependent upon the concentration of the enzyme The original test, as described by Astrup and Müllertz,[1] consists of placing a drop of the test solution on an unheated fibrin film, incubating for 18 to 20 hours at 37°C., and then measuring the size of the clear area (digested fibrin) However, since conventional fibrinogen preparations contain adsorbed profibrinolysin, activators of profibrinolysin, if present in the test solution, indirectly affect the size of the digested area Thus the test with unheated fibrin does not distinguish between the active proteolytic enzyme and an activator of the proenzyme

Profibrinolysin is heat labile Using this property of the proenzyme, Lassen[2] heated fibrin plates and found that the fibrin, following the heating procedure, is capable of being digested by fibrinolytic enzymes. Thus the area of lysis on heated fibrin plates results only from the action of already activated proteolytic enzymes in the test sample and is independent of activators in the sample being tested

The two tests, involving unheated and heated fibrin plates, can be used to demonstrate that all of the activity in the test sample results from activator, for if only activator is present, lysis will be obtained on the unheated

fibrin plate but not on the heated fibrin plate. It is not possible to use these tests as presently constituted to demonstrate that only fibrinolysin is present in the test sample or the absolute ratio of fibrinolysin to activator. However, if demonstrated by other means that only fibrinolysin is present in the test sample, its concentration can be estimated through measurement of the size of the lysed area.

Reagents and Apparatus· Fibrinogen. A source of relatively pure fibrinogen is required. Astrup and Mullertz recommend fibrinogen prepared from oxalated ox blood which is treated with tricalcium phosphate and the fibrinogen precipitated with ammonium sulfate.[1] The fibrinogen prepared by the free-thaw technic of Ware et al [3] may also be used. The preparation should be buffered at pH 7.8 with diethyl barbiturate buffer and the ionic strength of the solution used to form the fibrin film should be 0.15. The concentration of the fibrinogen in the solution to form the fibrin can be 0 1 per cent or 0.2 per cent; the higher concentration of fibrinogen produces the more stable fibrin film

Thrombin A source of relatively pure thrombin is required. Astrup and Mullertz[1] in their original experiments used thrombin obtained from Løven kemiske Fabrick, Copenhagen. Thrombin Topical, Parke, Davis & Co., Detroit, is also satisfactory The powder is dissolved in a 0.9 per cent NaCl solution to give a preparation with 100 N I.H. units/ml

Buffer. Astrup and Mullertz[1] recommend the use of diethyl barbiturate buffer prepared by adding 662 ml of 0.1 M sodium diethyl barbiturate to 338 ml of 0 1 M HCl and diluting with 320 ml. water. The pH should be 7.8 and the ionic strength 0.05. The ionic strength of the solution used to form the fibrin must be low and constant, and to insure low ionic strength, fibrinogen solutions of relatively high ionic strength must be diluted with the low ionic strength buffer to bring the final ionic strength to about 0.15.

Petri dishes: Dishes of 10 cm diameter are used Bottom and cover should be even so that they can be stacked in an incubator The dishes are heat-sterilized before use in the test.

Procedure Nine ml of the diluted fibrinogen solution (0 1 or 0 2 per cent, pH 7 8, μ = 0 15) is pipetted into each horizontal petri dish and clotted with 0.2 ml of thrombin solution. Unheated fibrin plates are ready for the addition of the test solution as soon as clotting has occurred Petri [illegible] addition of the test solution

Solutions to be tested (0 030 ml.) are added in a small drop to the surface of the fibrin in the petri dishes from a 0.1 ml pipette, graduated in 0 001 ml Three separate drops can be placed on each plate. The plates are incubated for 18 to 20 hours at 37°C.

Calculations The digested areas are estimated by measuring two perpendicular diameters of each cleared, circular area Measurements of areas which are not circular give less accurate results The product of the perpendicular diameters in square millimeters is obtained and the mean of three determinations of aliquots of the test sample are calculated. The areas can be converted to concentrations by interpolation on a reference curve which is obtained through the testing of dilutions of activators or fibrinolysin of known concentrations on the fibrin plates. Astrup and Müllertz plot the log of concentration of fibrinolysin as the abscissa, with the product of the diameters as the ordinate, and obtain a straight line.

If only an activator of profibrinolysin is present in the test sample, the unheated plates but not the heated plates will show cleared areas following incubation. Only semi-quantitation of the activator is possible unless the nature of the activator is known and its concentration or activity can be determined by some other method If such information is available or can be obtained, dilution curves using the fibrin plate method can be established for a specific activator

Precautions and Notes: 1. Unless the procedures are precisely followed a satisfactory fibrin film is not usually obtained. 2. The purity of the fibrinogen preparation is important. 3. A low ionic strength in the fibrinogen solution, which is carefully controlled, must be used. 4. The petri dishes must have a uniform bottom surface and they must be exactly horizontal at the time the fibrin forms Otherwise the thickness of the fibrin film will vary and, with variation in thickness, measurement of the area is without quantitative meaning 5 Heated fibrin has an altered susceptibility to proteolysis As pointed out by Lassen, two changes are probably pertinent: (a) heated fibrin is less susceptible to proteolysis than unheated fibrin, and (b) inhibitors of fibrinolysis associated with the fibrin are in part inactivated Because of these somewhat variable alterations in the susceptibility of heated fibrin to proteolysis, a quantitative comparison of lysed areas on unheated and heated fibrin plates cannot be justifiably made 6 Astrup and Alkjaersig[3] have pointed out that the contamination of the test samples with inhibitors greatly affects the results obtained with the fibrin plates, because inhibitors influence the stability of the enzymes during the incubation

References

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2 Lassen, M. Heat denaturation of plasminogen in the fibrin plate method. Acta Physiol scandinav 27·371, 1952

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2. Euglobulin Lysis Time

D. R. CELANDER and M. M. GUEST

Object: The euglobulin lysis time is considered to be an indicator of spontaneous fibrinolytic activity in plasma rather than a measure of an activator or proactivator. This assumption is based upon observations that such fractions regularly contain proteolytic activity against substances which appear to be free of profibrinolysin (plasminogen) such as specially treated casein and the synthetic substrates, TAMe and LEe. The supernatant portion of such fractions (whether obtained by lowering the ionic strength, adjustment of pH to 4.5 with acetic acid, precipitation with protamine sulfate or precipitation with CO_2) are found to possess essentially the same proactivator activity as does the original plasma. Furthermore, in plasmas from different subjects, little correlation is found between the euglobulin lysis time and assays which are presumed to measure primarily activators

Principle. When CO_2 is bubbled through human plasma, a precipitate forms This precipitate can be dissolved in phosphate buffer at pH 7 2 and the contained fibrin clotted with thrombin The clot which forms is observed for lysis at 37°C. The time required for lysis is reasonably reproducible in aliquots of a given plasma, but varies with plasmas from different human subjects It is presumed that the test measures the amount of fibrinolytic activity which can be spontaneously generated

Reagents and Apparatus

Carbon dioxide A tank of CO_2 is fitted with a valve permitting fine control of rate of flow. This valve is fitted with plastic tubing which in turn is connected to a piece of glass tubing drawn to a fine capillary tip

Thrombin, fibrinogen, imidazole and phosphate buffers are prepared as described for the urokinase assay (method of Celander and Guest see p 273).

Plasma is separated from blood collected into anticoagulant (either citrate or oxalate) in the ratio of 9 volumes of blood to 1 volume of anticoagulant by centrifugation at approximately 2500 g

Procedure A 0 4 ml aliquot of the plasma is diluted to a total of 8 0 ml. by addition of 7.6 ml distilled water (i e, a 1 20 dilution) Carbon dioxide is then bubbled into the solution for 15 to 30 seconds through the capillary tube described above.

The precipitate which forms is collected by centrifugation at 2500 g for 15 minutes. The supernatant portion is discarded and the precipitate

is redissolved as quickly as possible in M/15 phosphate buffer, pH 7.2, transferred to a 10 x 75 mm. tube, clotted with "rod thrombin" (see urokinase assay) and observed for lysis at 37°C. The time required for lysis is recorded.

Occasionally material from human patients is encountered in which the fibrinogen titer is so low or the fibrinolytic activity so high that the redissolved euglobulin precipitates fail to clot. Such solutions are mixed with equal parts of bovine fibrinogen (0.1 per cent) in a total volume of 0.4 ml., clotted and observed for lysis at 37°C. A similar problem also arises when fibrinolytic activity is experimentally generated in animals. If overt fibrinolytic activity is expected through experimental manipulations, control samples are also tested for their activity against a bovine fibrin clot in order that they may be compared with the experimental samples.

Precautions and Notes

1. No attempt has been made to quantitate in units the activity observed in this assay. In the normal, unstressed individual, the clot formed from the individual's own fibrinogen ordinarily requires from 1 to 3 hours before lysis occurs.

2. The mixing of the redissolved euglobulin precipitate with bovine fibrinogen results in a clot (especially in samples drawn from individuals with a comparatively normal titer of fibrinogen) in which there is considerable lengthening of the lysis time when compared with that of a clot formed from the euglobulin precipitate alone. The reasons for the lengthening in lysis time are that in such a system the enzyme solution is diluted 1.2 and the amount of fibrin to be lysed is greater. On the other hand, the presence of bovine fibrinogen is an indication that the system does not measure activator to any appreciable extent, bovine fibrinogen carries with it adsorbed profibrinolysin which, if activated, would result in a reduction in lysis time.

3. Fibrinolytic Potential Assay

M. M. GUEST and D. R. CELANDER

Object The assay is designed to measure, by means of an in vitro system, the potential for fibrinolytic activity in circulating blood.[1] It has been found that the measured level of activity is related to the availability of activators of profibrinolysin (plasminogen). Available evidence indicates

that the data obtained may also be an indication of whether or not some of the fibrinogen is free of adsorbed profibrinolysin; if fibrinogen, lacking adsorbed profibrinolysin, is present in plasma, the curve representing the rate of fibrin dissolution does not reach the base line. The validity of the assay is not limited to the human subject; reproducible data are also obtained when other animal species are studied.

Principle: The assay is based upon the observation by Fearnley and Lackner[2] that fibrinolytic activity can be demonstrated in diluted plasma from normal human subjects if the blood is immediately cooled during its removal from a vein and if it is kept near 0°C. during centrifugation and dilution procedures. In addition in the method described, reducing the pH of the blood to about 6.4 (and maintaining the pH of plasma at this level during processing) greatly enhances the fibrinolytic activity which is measured.

After processing the plasma at pH 6.4 and at 0°C., the fibrinogen in the diluted plasma is converted to fibrin by the addition of thrombin. The fibrin is wound onto abraded glass rods and incubated in phosphate buffer, pH 7.7 at 37°C. Rods are removed from the phosphate buffer at intervals, washed with saline, and the residual fibrin is hydrolyzed. The tyrosine in the hydrolysate is measured by the method of Folin and Ciocalteau. A plot of residual fibrin against time permits estimation of the time at which one-half of the fibrin has been lysed. Fibrinolytic potentials are evaluated by comparing half-times.

Reagents

Anticoagulant-buffer mixture:

5.75 ml. 1 M KH_2PO_4
4.25 ml. Distilled H_2O Total 10 ml
10 ml. 4 per cent Na_3 citrate

Mix equal quantities of the phosphate and citrate solutions. The mixture may be stored at 4°C. Use 2 ml. anticoagulant-buffer mixture to 10 ml. blood (1·6 dilution)

Phosphate buffer, pH 6.4 (for diluting plama)

29 ml. M/5 Na_2HPO_4
71 ml. M/5 KH_2PO_4
200 ml. distilled H_2O

Phosphate buffer pH 7.7 (for 37°C. incubation)

179 ml. M/5 Na_2HPO_4
21 ml. M/5 KH_2PO_4
400 ml. distilled H_2O

Sodium hydroxide, 0.1 N.; Na_2CO_3, 15 per cent; tyrosine standard, 0.1 mg./ml. (aliquots are stored at −20°C.), Folin-Ciocalteau reagent, 1 part reagent to 5 parts H_2O; Thrombin Topical (Parke-Davis)—100 N.I.H. units/ml.; saline, 0.9 per cent NaCl (for washing fibrin following the incubation)

Apparatus: Refrigerated centrifuge, water baths, various glassware, Klett colormeter with red filter or Coleman Jr. spectrophotometer.

Rods for winding out the fibrin are prepared from glass rods 130 mm. long and 1.5 to 2 mm. in diameter. One end of each rod (about 40 to 50 mm.) is abraded by rubbing with emery cloth. The abraded portion of the rod provides a surface to which the fibrin will adhere as it is wound onto the rod.

Procedure

1. Use syringe, cooled to 0°C., to withdraw blood from subject. Seven and one-half ml. of the blood are immediately transferred into a 12 ml. graduated centrifuge tube containing 1.5 ml. of the anticoagulant-buffer mixture. Mixing is accomplished by stoppering the test tube and inverting several times. The tube is then returned to the ice bath. Sufficient blood-anticoagulant-buffer mixture is removed from the test tube for hematocrit determination (Wintrobe).
2. The remaining blood-anticoagulant-buffer mixture in the test tube is transferred to a 15 ml. plastic centrifuge tube and centrifuged at 8000 r.p.m. for 7 to 9 minutes (angle head) in a refrigerated centrifuge, cooled to 0°C.
3. Transfer centrifuge tube to ice bath All test tubes and reagents are cooled to 0°C in the ice bath during the diluting and aliquoting procedures. Remove plasma by pipette and transfer to another test tube. Transfer 3 ml of the plasma to a tube containing 33 ml of the pH 6 4 phosphate buffer and mix well Transfer a 2.4 ml. aliquot of this mixture to each of 12 test tubes (13 x 100 mm.)
4. The fibrinogen is converted to fibrin while the test tubes are in the ice bath. To each of the 12 test tubes containing the 2 4 ml aliquots of diluted plasma, add 0 1 ml of 100 N.I H units/ml. thrombin. Mix the thrombin with the plasma by stirring briefly (10 seconds) with an abraded glass rod Leave the rod in the test tube and allow 30 minutes for the fibrin to form Carefully wind the fibrin onto the abraded end of the glass stirring rod
5. Transfer three of the rods to the saline wash (*see* 7). Transfer the remaining rods individually to 13 by 100 mm. test tubes in a 37°C. water bath Each test tube contains approximately 5 ml. of the

pH 7.7 phosphate buffer. (The buffer is added to the tubes sometime previously so that the temperature of the buffer is equilibrated at 37°C.)

6. Transfer the rods at intervals of 15 minutes (or less, if the activity is expected to be high) from the pH 7.7 buffer in the 37°C. bath to the tubes containing the saline wash, i.e., the first rod is transferred at 15 minutes, the second rod at 30 minutes, the third rod at 45 minutes, etc.
7. The saline washes consist of approximately 5 ml. of 0.9 per cent NaCl in 13 x 100 mm. test tubes. The rods are washed at 0°C. for a total of 45 minutes with transfer of the rods to tubes containing fresh saline every 15 minutes.
8. At the completion of the saline wash, transfer the rods individually into 13 x 100 mm. test tubes containing 2 ml. of 0.1 N NaOH. After all rods and their fibrin have been washed and transferred to the NaOH solution, hydrolyze for 3 minutes by placing the tubes in a rack in a boiling water bath. Stirring the rods in the NaOH solution helps to insure complete hydrolysis. If there is insufficient time to complete the assay, the tubes may now be covered and stored at 4°C. until the next day. This is the only point at which the assay can be stopped.
9. The Folin-Ciocalteau assay is performed as follows. To each tube add 3 ml. of 15 per cent Na_2CO_3 and 1 ml. of the Folin-Ciocalteau reagent, diluted 1 to 6. Mix well with a stirring rod and allow 30 minutes for color development. Read on Klett colormeter with a red filter (620 mμ) or Coleman Jr spectrophotometer at 620 mμ.

   A tyrosine standard is also measured. To 0.2 ml of the tyrosine standard (frozen aliquot), add 1.8 ml of 0.1 N NaOH, 3.0 ml of 15 per cent Na_2CO_3 and 1.0 ml. of the Folin-Ciocalteau reagent (1 to 6 dilution). Allow 30 minutes for color development and read as above.

   Blanks consist of 2.0 ml 0.1 N NaOH, 3.0 ml 15 per cent Na_2CO_3 and 1.0 ml of the Folin-Ciocalteau reagent (1 to 6 dilution).

Calculations Milligrams of tyrosine determined are converted to milligrams of fibrinogen (fibrin) by multiplying milligrams of tyrosine by 10. The concentrations of fibrin are then plotted on graph paper as the ordinates and the times of incubation at 37°C as the abscissae. From this curve, the time at which one-half of the fibrin has disappeared is determined, this is the "fibrin half-time" and is useful as a measure of the fibrinolytic activity present in the plasma sample. A short half-time indicates a high fibrinolytic

potential (probably due to the activator concentration) and a long half-time indicates a low fibrinolytic potential.

Normal Range of Values: Man: Half times of 50 to 90 minutes;[3] dog: 10 to 70 minutes.

Precautions and Notes

1. Buffers, other than phosphate, have been found to reduce the fibrinolytic activity which is measured. 2. The assay must be carried through to hydrolysis of the residual fibrin in sodium hydroxide on the same day that the blood is drawn. 3. It is important to maintain the low temperature during processing as described in the procedure. 4. Winding the fibrin onto the glass rods requires practice; if results are to have quantitative significance, all the fibrin from an aliquot must be transferred to the rod and it must be wound in a thin, even film. 5 If residual fibrin remains on the rod after several hours of incubation at 37°C. and several sequential determinations show no diminution of fibrin, it appears to indicate that some of the fibrin was essentially free of adsorbed profibrinolysin.

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4. The Thrombolytic Activity of Plasma (Isotopic Method)

A. P. FLETCHER

A. MEASUREMENT OF THROMBOLYTIC ACTIVITY

Object of the Test To determine the thrombolytic activity (clot-lysing properties) of plasma under as nearly physiologic conditions as possible Under ordinary circumstances this procedure is an assay for plasminogen activator in plasma but the procedure can be modified (*vide infra*) to permit the measurement of other plasminogen system components and is also of great utility for use with fluids other than plasma.

Principle.[1] Plasma to be assayed for plasminogen activator is incubated with a previously prepared substrate clot, which is trace-labeled with I^{131}-tagged fibrinogen and is of defined plasminogen concentration After a standard incubation period, the residual unlysed clot is removed and the degree of clot lysis determined by assay of the plasma supernatant for radioactivity.

Because dilution of the assay plasma sample is minimal, the method measures the over-all effective rather than the actual concentration of plasminogen activator, i e., that concentration available for clot lysis after inhibition by various plasma moieties has occurred. This principle of minimal sample dilution or other sample alteration, prior to or during the assay procedure, is one of great importance because of the complex nature of plasminogen system interaction in plasma. Procedures which involve system dilution or partial isolation of plasminogen system components, though valuable for specialized purposes, disturb the complex balance between individual components and may yield results of invalid or uncertain physiologic significance.

Using plasminogen-poor clots[1] the method becomes insensitive to the presence of activator, but the substrate still retains sensitivity to the action of the enzyme plasmin On the other hand, by increasing the proportion of clot plasminogen, and forming clots with a high surface/volume ratio,[2] the method can be made sufficiently sensitive to plasminogen activator to detect its presence at the very low concentrations occurring in the plasma of resting man

The first procedure (designated procedure 1 in later sections) is optimal for the detection and quantitation of enhanced plasma thrombolytic activity and its main use has been to monitor the biochemical changes occurring during thrombolytic therapy.[3] However, the technic is also useful for the study of pathologic plasma proteolytic states arising secondarily to disease processes[4] The second method of clot preparation (designated procedure 2 in later sections), more briefly described, provides a substrate clot and modified assay method for the quantitation of very low levels of plasma thrombolytic activity

Reagents and Apparatus 1. I^{131}-labeled fibrinogen (see separate section, B. for the preparation of this reagent)

2 A "standard" bank plasma A large volume of this reagent is divided into small tubes and stored at −30°C., tubes being unfrozen as required

3 Plasminogen purified by the Kline or other acid extraction procedure. This reagent usually exhibits excellent stability for months, if stored at pH 2 0 and 2°C ; however, it is advisable to check the standardization of this preparation every 2 weeks by means of the casein plasminogen assay.

4. Nichrome wires, 26 S.W.G., coiled into spirals at their lower ends, to fit 8 x 80 mm. serologic tubes.

5. A well-type gamma scintillation counter and scaler. A single channel pulse height analyzer is a most useful accessory for procedure 1, and its employment for low level counting required for procedure 2 is essential. *Automatic gamma counting equipment with result print-out greatly facilitates large-scale work.*

Procedure 1. Preparation of I^{131}-labeled substrate clots: One to four lambda of iodinated fibrinogen (1-2 per cent solution) and 1 casein unit of plasminogen are added to 0.5 ml. plasma in an 8 x 80 mm. serologic tube. The quantity of added radioactivity is calculated to give 100 c.p.m per μg. of fibrinogen. A spiral nichrome wire is inserted into the tube and 0.1 ml. thrombin (50 units/ml.) is added. The tubes are incubated for 1-2 hours in a 37°C. water bath and after retraction are withdrawn by means of the wire Approximately 50 such clots are suspended, by means of the wire, around the edge of a 2 L beaker and washed in normal buffered saline, overnight, at 2°C. This procedure reduces blank radioactivity to low and consistent values.

If clots are to be prepared in bulk, it is convenient to perform the initial mixing of reagents (plasma, iodinated fibrinogen and plasminogen) in a single beaker and pipette volumes of this mixture into individual tubes. In this case it is essential that the beaker should be well and continuously stirred (magnetic stirrer) as otherwise the plasminogen suspension will be unevenly distributed between the individual tubes.

Procedure 2. Plasminogen enriched highly sensitive substrate clots: Two-tenths ml. plasma, 0.4 casein units plasminogen and sufficient iodinated fibrinogen to give 150-200 c p.m./μg. fibrinogen are clotted with 0 1 ml thrombin (thrombin, 10 units/ml) These clots are formed in glass tubes of 7 mm O D. (sealed at one end) by rotation of the tube in an horizontal plane at approximately 350 r p m in the chuck of a geared electric motor. Clots formed in this manner are small, compact and largely free of entrapped plasma, a critical feature of the technic. When fully formed, the clot is floated from the tube with buffered saline (0 1 M PO_4, pH 7 6, and 0.9 per cent NaCl) and washed overnight in this fluid by gentle tube agitation.

Procedure 1: Thrombolytic assay method· Patient blood specimens, taken in standard oxalate tubes, are immediately chilled in ice and the plasma separated in a refrigerated centrifuge (2000 r p m. for 5 minutes) It is desirable to perform the thrombolytic assay as soon as feasible after specimen collection, but in any event within a 2-hour period, the specimen being maintained at 2°C. till assay

One-half ml. of the plasma specimen is incubated in a 10 x 100 *mm.* tube with a single prepared clot for 30 minutes at 37°C. The clot is withdrawn by means of the wire, removed from the wire and centrifuged in 2 ml. normal saline; this supernatant is added to the residual plasma and the total radioactivity is determined. Blank values for clots incubated in saline and for completely lysed (lysed with ficin or other proteolytic enzyme) clots are also obtained.

Procedure 2 for low activity determinations: Single clots prepared as in procedure 2 (*above*) are drained of wash fluid and incubated with 0.2 ml. patient plasma for 2 hours at 37°C. The unlysed clot is separated by filtration under air pressure (10 p.s.i.) using 5 ml. capacity funnels fitted with medium porosity-sintered glass discs. An additional 2 ml saline is used as a clot and filter wash, and the total filtrate is assayed for radioactivity. Blank values for clots incubated in saline and aged plasma are run for control purposes.

Scrupulous care must be exercised in the cleaning of the filter funnels to avoid radioactive contamination in subsequent runs and, after use, it is advisable to incubate the funnels for 6 hours in a papain solution to remove protein-bound radioactivity.

Expression of Results. Clot-specific radioactivity is determined for each set of clots (corrected count for fully lysed clot/μg clot fibrin), and consequently it is convenient to express results as μg. fibrin lysed from the assay clot during the standard incubation period by the plasma sample.

After correction of counts for background, the results are calculated as follows:

$$\frac{\text{specimen count}}{\text{lysed clot count}/\mu g \text{ fibrin in clot}} = \mu g. \text{ fibrin lysed by plasma specimen}$$

When measuring high activities with procedure 1 clots, pulse height analysis (which greatly reduces radioactive background correction) and low blank radioactive correction, the total corrections may be insignificant, under these conditions, it is sometimes advantageous, especially in in vitro work, where relative rather than absolute figures are of significance, to express the results as percentage clot lysis $\left(\frac{\text{specimen count}}{\text{lysed clot count}} \times 100\right)$. Alternatively, under these circumstances, it is sometimes sufficient to express relative results in terms of actual counts

Normal Values: The adaptability of this technic, and the fact that procedure 1 is designed to measure the enhanced plasma thrombolytic activity *developing under unusual circumstances (either therapeutically induced or* pathologic), precludes more than a brief statement of normal values, how-

ever, the technic is a well established one and many studies with it have appeared.

Procedure 1 is unsuitable for the assay of resting thrombolytic activity, but during intensive streptokinase therapy[1,3] the procedure will yield values of 100-1000 μg. fibrin lysed/30 minutes (values several hundredfold the resting level); the infusion of urokinase into patients may also produce approximately the same values Similarly, when used for the diagnosis or study of pathologic plasma proteolytic states, the assay will usually demonstrate increased levels of plasminogen activator[4] usually 2-20 fold increased over normal, but occasionally greater.

On the other hand, procedure 2[2] is specifically designed for the assay of plasma thrombolytic activity at low levels and the normal uncorrected range for resting man is 2.7-8.1 μg. fibrin lysed/2 hours However, significant corrections have to be applied for method blank due to the spontaneous lysis of clots incubated in saline and aged plasma (respectively averaging 2 and 3 6 μg. fibrin); these corrections indicate that the average value for plasma thrombolytic activity, measured under these circumstances is 1.4-3 μg. fibrin lysed/2 hours. Use of this method in patients, with various disease syndromes, has yielded up to 200 μg. fibrin lysed/2 hours, but usually the values are considerably lower with wide variation in the different patients The method, at least in its present form, is unsuitable for investigation of disease situations in which decreased thrombolytic activity is inferred to be of etiologic importance.

Precautions and Sources of Error: Though only low concentrations of radioactive material are employed, the precautions customary in all isotopic assay work should be enforced.

The assay designated procedure 1 (once experience in its use has been gained) presents few difficulties, is of great versatility and utility, and readily yields highly reproducible results. However the procedure 2 assay, because of the low activities being assayed, requires both considerable experience in its use and considerable care in its performance before satisfactory results are attainable.

B Method for Iodination (I^{131}) of Fibrinogen (Based on the Eisen and Keston Method[5])

Reagents. (1) 20 mc. of I^{131}. (2) IRA-400 (Cl) Amberlite resin. (3) 0.02 M potassium iodide (4) 2 N hydrochloric acid (5) 2 N sodium hydroxide. (6) 1 M sodium nitrite (7) 1 M ammonium sulphamate. (8) 0.1 M borate buffer, pH 8.0 (9) 0.1 M phosphate buffer with 0 9 per cent NaCl pH 7.6. (10) Bovine fibrinogen (92-95 per cent of the nitrogen being clottable by thrombin)

Note: (a) Sodium nitrite is unstable and must be made up fresh each occasion. (b) Bovine fibrinogen is freshly dissolved on each occasi in 0 1 M borate buffer. Four ml of 5 per cent solution is prepared. (c) 1 x 30 cm. chromatography column is used for the IRA-400 resin. A cm. column is formed in the usual manner and is equilibrated with t phosphate saline buffer. The column is stoppered and used in stage 9 the procedure.

Steps in Performance of the Procedure: (1) Transfer 20 mc. of I to a small beaker. Add approximately 1 ml. of water to the original cc tainer and add washings to beaker. Add 100 lambda 0.02 M KI. T the reaction (sample withdrawn with a small Pasteur pipette and tested pH paper), which should be alkaline; if not, add a small quantity of 2 NaOH until this is achieved.

(2) Heat beaker gently on an electric hot-plate until the volume reduced to approximately 200-500 lambda Remove from hot-plate a allow to cool.

(3) Add sufficient 2 N HCl until the pH is below 2. Test pH. Tl usually requires 150-200 lambda.

(4) Add 50 lambda of 1 M sodium nitrite. The solution will becor brown.

(5) Add slowly 50 lambda of 1 M sulphamate Gas will be evolv

(6) Add sufficient 2 N NaOH to raise pH to approximately 7-8. Tl usually requires 150-200 lambda.

(7) Add fibrinogen from pipette with bulb and mix rapidly.

(8) The test pH should be approximately 8 Wait 15 minutes.

Note: Each step in procedure 1-8 should be completed l mixing of the reacting substances for 30-60 seconds.

(9) The mixture from step 8 is then passed 4 times through the IR 400 resin column. About 8 ml of phosphate saline buffer is used as a was

(10) Add 0.2 ml heparin (1 mg /ml.) to the solution and store in lead container at 2°C

(11) The fibrinogen solution is assayed for radioactivity and percenta tyrosine clottable by thrombin.

Precautions. This method should not be attempted by persons u trained in the use of isotopes. Minimum equipment required is lead shiel ing, fume cupboard, isotope storage and disposal facilities, simple remc handling apparatus, survey meter and badge service

In addition, use and standardization of this substrate requires a we type gamma scintillation detector and suitable scaler preferably equipp with a single-channel pulse height analyzer.

Comments: If undiluted I^{131}, rather than the usually supplied diluted form, is ordered and precautions taken to limit step 1 volumes between 200-500 lambda, step 2 of the procedure may be omitted.

If fibrinogen of higher specific radiocativity is required (the usually prepared specific activity is suitable for many purposes[1,3]), the amount of I^{131} employed may be increased or, more conveniently, the quantity of carrier KI used in step 1 may be increased from 100 to 200 lambda with doubling of the sodium nitrite and sodium sulphamate volumes used in steps 4 and 5. However, this latter procedure, while increasing specific protein radioactivity, also results in substantial increase in the degree of non-isotopic protein iodination which, for some purposes, may be undesirable.

Step 9 in the procedure describes the removal of unbound I^{131} by ion exchange on an IRA-400 column. Alternatively, unbound I^{131} may be removed by gel filtration through a G-25 Sephadex column (Pharmacia, Upsala, Sweden) and depending on the use to which the final product is put, this alternative step is sometimes useful.

The half-life of I^{131} is 8 days but, as prepared above, the material is most often usable for 2 weeks provided that appropriate adjustment for radioactivity decay is made. Work is now in progress to substitute I^{125} for I^{131} in the procedure as this latter isotope, though very expensive, has a half-life of 60 days The use of fibrinogen labeled with I^{125} would remove much of the inconvenience and waste inseparable from the use of a labeled substrate bearing a short-lived isotope.

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5. The Standardized Serial Thrombin Time for Detection of Circulating Fibrinolysin*

W. O. REID

Object of the Method: The measurement of circulating excess proteolytic and thrombolytic enzyme (fibrinolysin)

Principle: Platelet-poor *oxalated* plasma is incubated at 26°C. or 37°C. and tested at intervals for evidence of fibrinogenolysis by the thrombin time. Thrombin time elevations at 1 or 2 hours at 26°C. or at 15 or 30 minutes at 37°C. are indicative of excess circulating fibrinolysin (plasmin) and a hemorrhagic diathesis.

Apparatus and Reagents: Twelve x 75 mm. clean glass test tubes; centrifuge (International Clinical Centrifuge Head No 809, RCF 1790 at 3,400 rpm); sterilized 10 cc. syringe and needle for venepuncture; test tube rack; Becton Dickinson black-topped tubes to hold 4.5 ml. of whole blood and 0.5 cc. of 0.1 M Na oxalate (B3206 Nax); human thrombin (Fibrindex, Ortho); 0.2 ml and 1 ml. pipette

Procedure. Collect 4 5 ml of blood in an oxalated vacuum tube (Vacutainer 3206 Nax, B-D) by means of nontraumatic venepuncture, and mix thoroughly but gently. Centrifuge immediately at 3000 rpm for exactly 5 minutes Promptly withdraw 1 4 ml of plasma without disturbing packed cells and distribute 0 2 ml aliquots in each of seven 12 x 75 mm. test tubes Place the rack in a water bath at 26°C or 37°C. and test at 0, 1, 2, 4, 6, 8 and 24 hours at 26°C., or 0, 15, 30, 45 or 60 minutes at 37°C as follows. add 0 2 ml of thrombin solution (Fibrindex, Ortho, 50-unit vial) dissolved in 1 ml of 0 85 per cent saline solution and tilt the tube gently through an arc of approximately 80°C from the vertical to the horizontal and back, at least 90 times a minute Record with a stop watch the time required for a coagulum to appear. Should no clot form in 4 minutes, record this and discontinue Plot the thrombin time in seconds against the incubation time in hours or minutes on a semilogarithmic scale.

Values Obtained The upper limits of normal at 26°C are 8 seconds at 0 hour, 10 seconds at 1 hour, 45 seconds at 2 hours and greater than 4 minutes at 4 hours A value between 30 seconds and 45 seconds at 2 hours is indicative of a borderline abnormality. The upper limits of normal at 37°C are 8 seconds at 0 hour, 8 seconds at 15 minutes, 20 seconds at 30 minutes, and greater than 4 minutes at 45 minutes

* Supported by a grant from the National Institute of Arthritis and Metabolic Diseases, United States Public Health Service

The upper limits of normal were established by running 100 normal controls

Precautions and Sources of Error: If any of the directions for the test are not followed closely, the thrombin time will be affected:

(1) The serial thrombin time will be artificially elevated if the volume of plasma is decreased; if the blood is obtained traumatically and is in the syringe for longer than 1 minute; if the blood is allowed to stand for over 1 hour; if it is centrifuged too long; if the temperature of the water bath is raised; if a tube of larger diameter is used; and if the thrombin is not fresh (more than 8 hours after opening vial).

(2) The serial thrombin time will be artificially depressed if the tubes are capped; if a tube with a smaller diameter is used; if a larger volume of plasma aliquot is used; if bovine thrombin is used; if water is added to the thrombin instead of saline; if the temperature of the water bath is lowered, and if citrate is used

Discussion· The standardized serial thrombin time gives a rapid, accurate determination of circulating fibrinolysin. It appears to differentiate activator from existing fibrinolysin[2] and therefore, when abnormal, detects *net excess fibrinolysin. The test follows closely clinical hemorrhagic* diatheses and has had predictive value in preoperative evaluation[5] The 37°C. test was designed for rapid detection of excess fibrinolysin in cases of emergency where a diagnosis of cause of hemorrhage is important for proper treatment, such as obstetrical and gynecologic emergencies and post-operative surgical hemorrhage, especially cardiac by-pass patients The 26°C test is slightly more sensitive and has proved valuable in the detection of abnormal fibrinolysin in malignancy, thrombocytopenia and liver disease.[6 7]

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6. Assay for Human Proactivator (Prokinase)

D. R. CELANDER and M. M. GUEST

Object: Human plasma contains a component which appears to react stoichiometrically with streptokinase to form an activator (kinase); this activator accelerates the conversion of profibrinolysin (plasminogen) to fibrinolysin (plasmin). The circulating level of the component of plasma, which has been called proactivator or prokinase, is not limiting when streptokinase is introduced into the circulation of the human subject. However, the concentration of prokinase is of interest in basic studies of the physiology of fibrinolysis Furthermore, evidence is available that profibrinolysin and/or fibrinolysin may be identical to plasma proactivator,[2] if this is true, the procedure described could be used to determine the total profibrinolysin and fibrinolysin in a human plasma sample.

Principle The enzymatic reaction in which profibrinolysin is converted to fibrinolysin in the presence of plasmin activator is rapid; the rate is dependent upon the concentration of the activator formed and the concentration of profibrinolysin The one-stage assay for proactivator[1] is based upon the following sequence of reactions:

(1) Streptokinase + plasma proactivator ⟶ plasma activator

(2) Profibrinolysin $\xrightarrow{\text{plasma activator}}$ fibrinolysin

(3) Fibrin $\xrightarrow{\text{fibrinolysin}}$ soluble products

The assay system is constructed in such a way that fibrinogen, profibrinolysin and streptokinase are present in constant but excess amounts Under these conditions the rate of lysis of a clot formed in the presence of a proactivator, streptokinase, and profibrinolysin is dependent only upon the concentration of proactivator at constant temperature and pH. In practice,

these conditions are achieved by mixing a fixed amount of fibrinogen which contains profibrinolysin, a fixed amount of streptokinase, and a highly diluted sample of the plasma under test, then clotting the mixture immediately with a constant amount of thrombin. Clots formed under these conditions undergo lysis. The time of lysis is proportional to and is taken as an index of the amount of proactivator present.

Reagents

Bovine fibrinogen, thrombin and buffers are prepared and used in the same way as described for the urokinase assay (method of Celander and Guest, see page 273).

Streptokinase· One vial of Varidase is dissolved in 0.9 per cent NaCl to give a concentration in the final solution of 10,000 units of streptokinase per ml. This is aliquoted and frozen immediately in 0.3 ml. to 0.5 ml aliquots Immediately before use, an aliquot is thawed and diluted 1:10 with 0.9 per cent NaCl

Plasma dilutions: Plasma in an ice bath is diluted 1:100 with 0.9 per cent NaCl and this solution is diluted 1:8 through 1:256. Two-tenths ml. of 0.2 per cent bovine fibrinogen is mixed in a 10 x 75 mm. test tube with 0.1 ml of diluted plasma and 0 1 ml of Varidase containing 1000 units/ml. of streptokinase. The mixture is clotted immediately with thrombin on the end of a 2 mm diameter glass stirring rod which had been dipped to a depth of 5 mm in the thrombin solution, the lysis time of the clot is determined at 37°C. by the tilt-tube method Triplicate determinations are performed on each of the plasma dilutions The lysis times obtained with each dilution are averaged and converted to units of activator by reference to the standard activator curve (prepared originally by dilution of a purified urokinase preparation[3]) Dilutions of plasma giving lysis times in the range of 25 to 45 minutes will follow this curve most closely.

Calculation· Unitage is usually best calculated from the 1:6400 to 1 12,800 dilutions. It should be noted that in converting from units per ml. of dilution to units per ml of plasma, the actual observed unitage should be multiplied by the dilution of the plasma and then by 2. This manipulation is required since the standard activator curve is set up to take into account only a 1 2 dilution of the activator solution in the assay system, whereas in the plasma proactivator system the enzyme solution is diluted 1.4 (0.1 ml. plasma dilution, 0 2 ml. fibrinogen, and 0.1 ml. streptokinase solution).

The unit of plasma proactivator is defined as that amount of material which yields one unit of activator when acted upon by streptokinase The activator unit employed is identical in size to the unit for urokinase (one-

stage assay); i e., it is that amount of activator which will bring about the conversion of sufficient profibrinolysin to fibrinolysin to cause the lysis in 10 minutes at 37°C. of a clot from 1 0 ml of a 0.1 per cent bovine fibrinogen solution buffered at pH 7.2 with phosphate or imidazole and isotonically equivalent to 0 9 per cent NaCl.

Because of the high activator content of most plasma samples, it has been found convenient to divide the number of activator units by 100, thus converting the expression of plasma proactivator activity to hectounits (1 hectounit = 100 units).

Precautions and Notes: 1. Although preparations of fibrinogen usually contain a relatively large and uniform amount of adsorbed profibrinolysin, the concentration of this component should be checked in each batch of fibrinogen used in the assay of proactivator Several preparations of fibrinogen are available which are essentially free of profibrinolysin 2. Only bovine fibrinogen or fibrinogen from a species other than the human can be used in the assay since human fibrinogen contains profibrinolysin and human profibrinolysin carries with it proactivator activity.

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7. Preparation of Profibrinolysin (Plasminogen) and Fibrinolysin (Plasmin) (Method of E. C. Loomis)

Adapted by M. M. GUEST

Object The enzyme prepared by this method is useful as a reagent for assays of antifibronolysins and it may also be used for the study of the general properties of fibrinolysin, but the purity is not sufficient for precise kinetic studies Profibrinolysin is a useful reagent in the assay of fibrinolytic activators

Principle Profibrinolysin is separated from serum by ammonium sulfate fractionation The precipitate containing profibrinolysin is redissolved

these conditions are achieved by mixing a fixed amount of fibrinogen which contains profibrinolysin, a fixed amount of streptokinase, and a highly diluted sample of the plasma under test, then clotting the mixture immediately with a constant amount of thrombin. Clots formed under these conditions undergo lysis. The time of lysis is proportional to and is taken as an index of the amount of proactivator present.

Reagents

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Plasma dilutions: Plasma in an ice bath is diluted 1:100 with 0 9 per cent NaCl and this solution is diluted 1:8 through 1·256. Two-tenths ml of 0 2 per cent bovine fibrinogen is mixed in a 10 x 75 mm. test tube with 0.1 ml. of diluted plasma and 0.1 ml of Varidase containing 1000 units/ml. of streptokinase. The mixture is clotted immediately with thrombin on the end of a 2 mm. diameter glass stirring rod which had been dipped to a depth of 5 mm. in the thrombin solution, the lysis time of the clot is determined at 37°C. by the tilt-tube method Triplicate determinations are performed on each of the plasma dilutions. The lysis times obtained with each dilution are averaged and converted to units of activator by reference to the standard activator curve (prepared originally by dilution of a purified urokinase preparation[3]) Dilutions of plasma giving lysis times in the range of 25 to 45 minutes will follow this curve most closely

Calculation· Unitage is usually best calculated from the 1:6400 to 1:12,800 dilutions It should be noted that in converting from units per ml. of dilution to units per ml of plasma, the actual observed unitage should be multiplied by the dilution of the plasma and then by 2. This manipulation is required since the standard activator curve is set up to take into account only a 1·2 dilution of the activator solution in the assay system, whereas in the plasma proactivator system the enzyme solution is diluted 1:4 (0.1 ml. plasma dilution, 0 2 ml fibrinogen, and 0.1 ml. streptokinase solution).

The unit of plasma proactivator is defined as that amount of material which yields one unit of activator when acted upon by streptokinase The activator unit employed is identical in size to the unit for urokinase (one-

Activation of proenzyme to fibrinolysin: If the active enzyme is to be prepared, the precipitate obtained with 29 per cent of saturation with $(NH_4)_2SO_4$ is dissolved in 100 ml distilled water, transferred to a separatory funnel and shaken about 5 minutes with 25 ml. $CHCl_3$. After allowing to stand for 20 to 24 hours at room temperature, the $CHCl_3$ layer is separated and discarded. The aqueous phase is dialyzed against cold running tap water as described above The remainder of the procedures are identical with those for the preparation of profibrinolysin.

Activity of preparations: Using the Loomis unit of fibrinolysin which is defined as the amount which will dissolve 1 ml of a 0 3 per cent fibrin clot in 120 seconds at pH 7.2 and 45°C. in an isotonic saline system buffered with imidazole, Loomis reports an average of eight preparations from beef serum gave 2 2 units per mg.

Precautions and Notes: 1. Some fibrinolytic activity is occasionally found in the preparations of profibrinolysin 2. Plasma may also be used as a source material for isolation of the proenzyme and enzyme. If plasma is used, each four liters are treated with 600 ml of $Mg(OH)_2$ cream. The $Mg(OH)_2$ and the adsorbed prothrombin are removed from the supernatant plasma by centrifugation for 10 minutes at 5000 rpm. However, fibrinolytic activity obtained from plasma by this method is about one-tenth that obtained from serum. 3 The advantage of the chloroform activation is that the final preparation is free of activator. The way in which chloroform brings about activation is uncertain and the degree of activation may be variable

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in distilled water, dialyzed and reprecipitated at the isoelectric point of the protein. The proenzyme is redissolved in 0 9 per cent NaCl at pH 7.2. The preparation may be shell-frozen and lyophilized.

If the active enzyme, fibrinolysin, is to be prepared, the proenzyme is subjected to treatment with chloroform just prior to the isoelectric precipitation step.

Reagents and Apparatus

NaCl, $CaCl_2$, $(NH_4)_2SO_4$, $CHCl_3$, HCl, NaOH (C.P.).

Visking "No Jax" casings, 29/32 inch; refrigerated centrifuge; glassware

Procedure

Preparation of serum· Serum is prepared from oxalated bovine or human plasma by the addition during vigorous stirring of a volume of 0 04 M $CaCl_2$ equal to the volume of plasma to be defibrinated Stirring is continued for 30 minutes after the fibrin separates, to allow for complete conversion of prothrombin to thrombin and for antithrombin (present in serum) to destroy the thrombin Fibrin which is not collected on the stirring rod is removed by centrifugation

Each lot of serum is tested and should respond as follows: (1) one ml. serum + 0 1 ml 100 U. thrombin → no clot. Therefore, all fibrinogen was removed. (2) Two-tenths ml serum + 1 ml. purified fibrinogen → no clot. Therefore, all thrombin destroyed. (3) One ml serum + 1 ml. 0 2 per cent fibrinogen + 0 2 ml purified lung extract → no clot. Therefore, all prothrombin was converted to thrombin which had been inactivated as demonstrated in (2)

Separation of profibrinolysin from serum. The serum is cooled to 5°C and a saturated solution of $(NH_4)_2SO_4$ added dropwise with constant stirring to 25 per cent of saturation. The precipitated solids are discarded. The supernatant portion is cooled to 0°C. and the $(NH_4)_2SO_4$ saturation increased to 29 per cent by the dropwise addition of saturated $(NH_4)_2SO_4$ with constant stirring The precipitate is collected by centrifugation of 5000 r.p m. for 3 minutes The supernatant fraction is discarded.

The precipitate is dissolved in 100 ml distilled water and dialyzed for 16 hours against cold running tap water in Visking "No Jax" casings. The precipitate which forms on dialysis is collected by a short fast centrifugation, as above, dissolved in 100 ml of 0 9 per cent NaCl, diluted to 1500 ml. with cold distilled water (to a specific resistance of approximately 400 ohms), cooled to 0°C., and adjusted to pH 7 0 to 7.2 with 0.1 N NaOH, shell-frozen and lyophilized.

8 6. (If a visible precipitate remains undissolved, adjust the solution to pH 9; centrifuge and discard the residue.) 10. Dialyze the solution against 0.25 M sodium phosphate, pH 6.0 for 10 minutes (a fine precipitate should appear). 11. At completion of the 10 minute dialysis, transfer the dialyzed suspension to a beaker and place in the refrigerator at 4°C. for 1 hour. 12. Centrifuge and discard the supernatant fluid. 13 Dissolve the precipitate in distilled water with the aid of a drop or two of N HCl Dispersion of clumps in the precipitate may be accomplished by using a glass stirring rod, especially before adding the drop or two of N HCl. Kline states that the proenzyme in this preparation is stable for at least 2 weeks at 4°C.

Kline has been able to obtain crystallization of the proenzyme of one preparation by again adjusting the pH following step 13 to 8.2. If crystallization occurs, the crystals may be collected by centrifugation; the crystals can then be dissolved in distilled water with the aid of a drop or two of N HCl and recrystallized by adjustment of the pH to 8 2 Unfortunately, crystals are not obtained with every preparation of Fraction III as the starting material.

The solution obtained in step 13 may be lyophilized and the dry powder stored in a desiccator

Activation of the proenzyme to fibrinolysin:[2] 1 Dissolve the lyophilized powder containing profibrinolysin in distilled water by the addition of a few drops of N HCl Adjust the concentration of the powder containing the proenzyme to 4 mg /ml by the addition of distilled water. 2. Adjust the pH of the solution to 6 8 by the dropwise addition of dilute NaOH while stirring 3 Add to the solution 5,425 units of streptokinase per mg of powder containing profibrinolysin 4 Incubate the mixture for 10 minutes at 37.5°C 5 Adjust the pH to 8 7 with N NaOH 6 Centrifuge and discard the precipitate. 7 Adjust the pH of the supernatant solution to 7.6 with N HCl 8. Centrifuge and collect the clear solution of fibrinolysin 9 Chill solution to 0°C in ice bath. 10 Add ice-cold 95 per cent ethyl alcohol dropwise with mechanical stirring until the alcohol concentration reaches 10 per cent by volume (The fraction precipitated at a concentration of alcohol of 10 per cent has the highest activity per mg of nitrogen, but material with activity is precipitated up to an alcohol concentration of about 20 per cent)

The alcohol precipitation procedure results in about a 3 5 increase in specific activity when all the active fractions are combined. The product is stable in the frozen state and it may be lyophilized

Precautions and Notes· 1 The profibrinolysin obtained is sometimes difficult to bring into solution at a neutral pH 2 The purity of the products appears to be dependent upon the lot of fraction III which is used as the starting material

8. Purification of Human Profibrinolysin (Plasminogen) and Fibrinolysin (Plasmin) (Methods of Kline[1] and Fishman and Kline[2])

Adapted by M. M. GUEST

Object: Purified profibrinolysin and fibrinolysin are valuable reagents in fibrinolytic assay procedures. Highly purified enzyme preparations are necessary for physical-chemical characterization and for kinetic studies. The physiologic aspects of the fibrinolytic enzyme system may be uncovered through parenteral introduction of purified enzymes and such preparations may have therapeutic value.

Principle: Salt, alcohol and isoelectric precipitation of profibrinolysin have proved to be of limited value because of the coprecipitating properties of the proenzyme. The method described, however, depends upon isoelectric precipitation, the resistance of the proenzyme to denaturation at both high and low pH, and the precipitation of the partially purified proenzyme in the presence of phosphate buffer at pH 6.0.

The conversion of the purified proenzyme to fibrinolysin is accomplished by treatment with an excess of streptokinase and the specific activity of the enzyme is increased by alcohol fractionation.

Reagents: H_2SO_4, NaOH, HCl, NaH_2PO_4, Na_2HPO_4, C_2H_5OH, streptokinase

Procedure

Profibrinolysin purification.[1] 1 To each Gm of human plasma fraction III (Cohn), add 20 ml of N H_2SO_4 and extract at room temperature for 10 minutes with mechanical stirring 2. Centrifuge resulting suspension at 2500 r.p.m. for 10 minutes; remove any floating lipids by means of a policeman. 3. Decant supernatant solution and add N NaOH while stirring by hand until pH 11 is reached 4 Immediately and with continued stirring, add N HCl from a pipette until the pH is brought to 5 3 5. Place the preparation in the refrigerator (4°C.) for a minimum of 3 hours; it may be stored overnight without affecting the results. 6 Adjust the pH of the suspension to 2 with N HCl from a pipette while stirring by hand. 7 Centrifuge for 1 hour (room temperature at 2700 r.p.m.), remove any floating lipids with a policeman 8 Carefully decant the supernatant solution containing profibrinolysin from the gelatinous residue (The proenzyme is stable to pH 2 5 for at least 1 week) 9. While stirring the solution containing profibrinolysin, add N NaOH dropwise until the pH is adjusted to

bottles, and the water is decanted and discarded. The casein is then washed with 200 ml. 95 per cent ethanol followed by 200 ml reagent grade ether. Repeat twice. After the last ether wash, the casein is spread over a 20-inch filter paper and allowed to dry at room temperature. One hundred Gm. of the dried casein is dissolved in 1 liter of hot (90°C.) 1/15 M, pH 7.4 phosphate buffer. This solution is frozen and stored at −20°C. in lots of 2 ml. For use, the casein solution is thawed and diluted to 1 per cent with phosphate buffer, 1/15 M, pH 7 4

Substrate: Blood bank plasma is used as the activation substrate. The use of new batches of aged plasma does not alter the results

Procedure: 1. Fresh or freshly frozen urine may be used Determine pH and specific gravity. 2. Dialyze mixture of 1 ml urine, 1 ml. plasma, and 19 ml. distilled water in rotation dialysis apparatus against running tap water at 4° to 7°C for 90 minutes (electrolytes are removed in this step, activation of profibrinolysin occurs and the enzyme inhibitor complex is dissociated) 3. Transfer dialysis bag with contents to beaker containing 1500 ml distilled water at 4°C. Lower pH of distilled water to 5.4 by bubbling CO_2 through it for 7 minutes (fibrinolysin is precipitated and most of the antifibrinolysins remains in solution) 4 Transfer the contents of the dialysis bag to a 50 ml. plastic centrifuge tube Centrifuge for 10 minutes at 1200 g. Discard supernatant, wipe the walls of the tube with cotton to remove fluid but do not disturb precipitate, dissolve precipitated euglobulins in 1 ml of 1/15 M phosphate buffer, pH 7 4

5 Prepare blank and test samples as follows a Phosphate blank—5.0 ml 1/15 M phosphate buffer, pH 7 4. b. Casein blank—1.0 ml phosphate buffer plus 4.0 ml. of 1 per cent casein dissolved in phosphate buffer c Test samples—0 6 ml. of the redissolved euglobulins plus 0 4 ml phosphate buffer plus 4 0 ml. of 1 per cent casein in phosphate buffer Mix by inversion. Divide each test sample and each blank into two aliquots 6 One aliquot of each blank and one aliquot of each test sample are used for non-incubated (zero time) controls Add 2 5 ml of 10 per cent T C A. to each of these tubes, mix by inversion and allow to stand for 10 minutes at room temperature. Filter through Schleicher and Schuell filter paper #576, discard precipitate, and use filtrate for color development (step no 8) 7. The other 2 5 ml aliquot of each blank and each test sample are stoppered and incubated at 37°C for 90 minutes to permit the fibrinolysin to hydrolyze the casein At the end of the 90-minute incubation, add 2.5 ml. of 10 per cent T C A to each tube and allow to stand 10 minutes at room temperature; filter through Schleicher and Schuell filter paper #576 and save filtrate for color development (step no 8)

8 Add in order to 2 0 ml of each T C A filtrate, 0 5 ml 10 per cent NaOH, 7 0 ml distilled water, 3 0 ml. 20 per cent Na_2CO_3 and 1.0 ml.

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9. Assay of Urokinase (Method of von Kaulla and Riggenbach)

Adapted by M. M. GUEST

Object: The assay[1] is designed to measure the concentration of urokinase (profibrinolysin activator) in urine.

Principle. Urokinase reacts enzymatically with profibrinolysin (plasminogen) by first-order kinetics to form fibrinolysin (plasmin). Profibrinolysin in citrated normal plasma is converted to fibrinolysin by urokinase. However, at least a part of the fibrinolytic activity which develops is masked by the antifibrinolysins (antiplasmins) of plasma. For this reason the activated profibrinolysin (fibrinolysin) is separated from antifibrinolysins of plasma by precipitation of euglobulins (containing the fibrinolysin) in a low ionic strength medium. Proteolytic activity is measured by the amount of casein hydrolyzed through the proteolytic action of the redissolved fibrinolysin The tyrosine released from casein with corrections for specific gravity of the urine is used as a measure of urokinase activity in the tested urine sample

Apparatus Rotation dialyzer; Beckman pH-meter, Model G, American Optical Comp T-S-Meter, 37°C water bath, carbon dioxide tank with reduction valves, "Visking" cellulose dialyzing tubing, 8/32 inch, Coleman Junior Spectrophotometer with 19 x 105 mm cuvettes #14-3-2C; Schleicher & Schuell filter paper #576, 9 cm diameter, various glassware

Reagents ACD-blood bank plasma aged for 3-4 weeks, then frozen in lots of 5 ml.; phosphate buffer, 1/15 M, pH 7.42, trichloracetic acid (T C.A.), 10 per cent, NaOH, 10 per cent, Na_2CO_3, 20 per cent; phenol reagent "Leitz", tyrosine standard solution; casein, purified.

Preparation of casein Approximately 200 Gm. of casein (reagent grade, Hammarsten) is washed with distilled water in 250 ml. centrifuge

10. One- and Two-Stage Assays for Urokinase

D. R. CELANDER and M. M. GUEST

Object. Urokinase, an activator of profibrinolysin (plasminogen), is present in the urine of all human subjects and all animal species in which the urine has been examined for its presence The concentration of urokinase in urine varies during different physiologic states and in some pathologic conditions. Thus a measure of the level of urokinase excretion may be helpful in differential diagnoses. In addition, urokinase is being used for the activation of profibrinolysin, both in basic studies and for use in thrombolytic therapy. The assays described are easily performed, they permit relatively good quantitation of urokinase activity, and they give reproducible results.[1]

Principle. Assays for urokinase depend on the ability of dialyzed urine or purified fractions from urine to effect the enzymatic conversion of profibrinolysin to fibrinolysin (plasmin). The amount of profibrinolysin converted per unit time is a function of the concentration of urokinase and is measured by the time required for the generated fibrinolysin to lyse a standard fibrin clot. In the two-stage assay, urokinase and a relatively pure source of profibrinolysin are mixed and incubated for 35 minutes Aliquots of the solution are then tested for activity against the standard fibrin clot. In the one-stage assay, generation by urokinase of fibrinolysin from the profibrinolysin contaminant of the bovine fibrinogen and thrombin reagents takes place simultaneously with the dissolution of the clot by the fibrinolysin generated

Reagents

Fibrinogen Fibrinogen is prepared by the method of Ware et al[2] and frozen in 0 5 ml. aliquots in concentrations ranging from 2 to 4 per cent clottable protein Immediately before use the fibrinogen is thawed and diluted with imidazole buffer, pH 7.25, to a final concentration of 0.2 per cent and ionic strength equivalent to that of 0 9 per cent NaCl

Thrombin. Parke-Davis Topical Thrombin is used The contents of a 5000 unit vial are dissolved in 2.5 ml of 0.9 per cent NaCl and stabilized by the addition of 1 ml of glycerol This thrombin preparation is stable for 60 to 90 days at refrigerator temperature The thrombin is added to the reaction-mixture containing fibrinogen by dipping a glass rod, 2 mm in diameter, to a depth of 5 mm in the solution of thrombin and using the rod to stir the reaction mixture. This procedure results in the addition of

phenol reagent (Folin-Ciocalteu). Mix by inversion, stopper and incubate for 15 minutes at 37°C. to develop the color. The color is stable for at least 45 minutes. 9. Transfer solutions to 19 × 105 mm. Coleman cuvettes. Set Coleman Junior spectrophotometer at 100 per cent transmission at 6500 Å against phosphate blank. Determine per cent transmission of casein blank (should read between 97 and 100 per cent transmission if casein has not deteriorated). Set machine at 100 per cent transmission against casein blank. Read per cent transmission of test samples.

Calculations and Expression of Results: 1. From a series of standard tyrosine solutions, determine per cent transmission and plot on semi-logarithmic paper with the per cent transmission as the logarithmic ordinate A straight line should be obtained between 0 and 0.08 mg. tyrosine. 2. Subtract amount of tyrosine in non-incubated controls from tyrosine obtained in incubated samples. Correct for variations in specific gravity of urines by substituting values in the relationship.

$$\frac{\text{net mg tyrosine released}}{\text{specific gravity} - 1.000} \times 10$$

The values obtained are in mg. of tyrosine released, corrected for specific gravity, von Kaulla and Riggenbach call their unit the specific gravity coefficient (sp.g.c.). The unit is based on the activity in 1 ml. of urine.

Normal Range of Values

Males 5 to 18 sp g.c /ml (12 individuals, 1st morning specimen)
Females· 1.4 to 23 sp g.c /ml (7 individuals, 1st morning specimen)

Precautions and Notes. 1. If the urine pH is not between 5.4 and 7.0, it must be adjusted to fall within this range unless the sample can be analyzed immediately. Within the range indicated the urine may be frozen for at least 48 hours without loss of activity 2 Urokinase is adsorbed on mucoproteins during storage. If the urine is stored for an appreciable length of time in the cold, the urokinase activity can be released from the mucoproteins by repeated freezing and thawing 3. Fecal and heavy bacterial contamination result in high values but minor contamination with blood does not grossly affect the results. If appreciable fecal or bacterial contamination is present, the specimen should be discarded.

Reference

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to fibrinolysin will bring about lysis of the standard fibrin clot in 2 minutes at 28°C.

Procedure

One-stage assay: Two-tenths ml. of a solution of urokinase (dialyzed urine or urine fraction) is mixed directly with 0 2 ml of 0.2 per cent bovine fibrinogen, the system clotted with thrombin added by the stirring rod technic, and observed for lysis at 37°C. as indicated in the two-stage procedure.

Calculations

One-stage assay: In this procedure, conversion of the bovine profibrinolysin to fibrinolysin and the action of the latter on the fibrin clot occur concurrently. When the logarithm of urokinase concentration is plotted against the logarithm of lysis time, a straight line is obtained with lysis times ranging from 10 to 70 minutes. The unit of urokinase, measured in the one-stage assay, is defined as the amount of urokinase which when present in 1 ml. of a 0 1 per cent bovine fibrin clot brings about lysis in 10 minutes at a temperature of 37°C and a pH of 7.2 in a system buffered with either imidazole or phosphate. Although the unit of urokinase activity is based upon the lysis time of a 1 ml clot, the 0 4 ml. clot has been employed routinely to conserve reagents By use of appropriate constants, activity against a 0 4 ml. clot can be expressed in terms of the unit based upon the lysis time of 1 ml clot In constructing the assay curve, adjustment is also made for the 1:2 dilution in the 0 4 ml assay system.[1]

Precautions and Notes

1 Both assays have limitations. The two-stage assay is not ideal quantitatively since activation of profibrinolysin does not stop at the end of the 35-minute incubation period However, it is especially useful in demonstrating the presence of activator The limitations of the one-stage assay system are (a) the presence of a contaminating protease can directly destroy the fibrin clot, (b) a larger excess of profibrinolysin than that normally adsorbed on fibrin would be advantageous in insuring that the only limiting factor is the concentration of activator. Usually the two assays give results which agree closely It must be noted, though, that the unit in which the results of the two-stage assay is expressed is equal to approximately 10 of the one-stage assay units.

2 The one-stage assay functions best at lysis times between 10 and 70 minutes Longer lysis times tend to be less accurate while shorter lysis times are undesirable since the profibrinolysin present in the reagents may

approximately 0 005 ml. of the thrombin solution or approximately 7 units of thrombin.

Buffers: Imidazole buffer is prepared by the method of Mertz and Owen by dissolving 1.72 Gm. of imidazole in 90 ml. of 0.1 N HCl, adjusting the pH to 7.25 and the final volume to 100 ml. Phosphate buffer, M/15, pH 7.2 to 7.4, is prepared by mixing appropriate volumes of 0.5 M solutions of sodium phosphate (dibasic) and potassium phosphate (monobasic) and diluting to 1 liter.

Human profibrinolysin: Profibrinolysin present in 2 ml. of human plasma is isolated by adding 0.5 ml. protamine sulfate solution (8 mg./ml.), adjusting the pH to 9, allowing to stand 2 hours, readjusting the pH to 7, and diluting with distilled water to a final volume of 40 ml. (approximately 1:20 dilution of original plasma). The precipitates are collected by centrifugation and stored at −20°C. Prior to use, the precipitates are resuspended to a total volume of 2 ml with phosphate buffer.

Urine or urine fractions for assay: All solutions to be tested for urokinase activity in which the ionic strength is not equivalent to 0 9 per cent NaCl or which contain ions or non-ionized substances inhibitory to the enzyme systems are first dialyzed against 50 volumes of M/15 phosphate buffer, pH 7 2, at 4°C. for 12 to 20 hours.

Procedure

Two-stage assay. In stage 1 of the two-stage procedure, a 2 ml. aliquot of the urine activator solution to be tested, or a dilution thereof, is used to resuspend the precipitate of human profibrinolysin This system, containing a relative excess of profibrinolysin, is incubated at 28°C for 35 minutes. At this point, a 0 2 ml. aliquot of the incubation mixture is mixed with 0 2 ml. of 0 2 per cent bovine fibrinogen in a 10 × 75 mm. test tube, clotted immediately with thrombin, and observed for lysis at 37°C. by the tilt-tube method. The lysis time is a measure of the amount of profibrinolysin converted to fibrinolysin and hence of the potency of the urokinase solution under test

Calculation

Two-stage assay The assay depends upon the availability of two reagents—a standardized profibrinolysin essentially free of inhibitory substances and a highly purified fibrinogen Under the conditions of the test, when the logarithm of the concentration of kinase is plotted against the logarithm of the lysis time, a straight line is obtained The unit of urokinase in the two-stage assay is defined as that amount which activates one unit of profibrinolysin at a temperature of 28°C and pH of 7 2 in a period of 35 minutes. The unit of profibrinolysin is the amount which when converted

11. Assays for Fibrinolytic Enzymes Based on the Use of Synthetic Substrates: Assay of Urokinase

S. SHERRY, N. ALKJAERSIG and A. P. FLETCHER

The urokinase assay depends on the ability of this enzyme to split acetyl-l-lysine methyl ester (AcLMe) into acetyl-l-lysine and methyl alcohol. The amount of methyl alcohol liberated from the synthetic substrate is measured colorimetrically by a modification of the method of Siegelman et al.[1] Acetyl-l-lysine methyl ester is the most sensitive of the urokinase substrates; it is 8 and 18 times more sensitive than l-lysine methyl ester (LMe) and tosyl-l-arginine methyl ester (TAMe), respectively At the present time, the assay is most useful as a biochemical standardization for concentrated urokinase preparations.

The method described for assaying urokinase can be adapted readily for the assay of plasminogen (SK activation) or plasmin, since acetyl-l-lysine methyl ester also is the most sensitive of the various plasmin substrates (2 5 and 4 times more sensitive than TAMe and LMe, respectively). The technic for quantitating methyl alcohol release is useful for measuring the hydrolysis of all arginine and lysine methyl esters used in the study of various fibrinolytic and coagulation enzymes; included among these substrates are the methyl esters of arginine (AMe), acetyl arginine (AcAMe), benzoyl arginine (BAMe), tosyl arginine (TAMe), lysine (LMe), acetyl lysine (AcLMe) and tosyl lysine (TLMe), the latter being the most sensitive thrombin substrate.

Reagents

Standard substrate: Acetyl-l-lysine methyl ester dried with phosphate buffer (pH 7 6) and NaCl * Twenty mg /ml in distilled water yields a solution containing 0 016 M substrate in 0 066 M phosphate buffer and 0 6 per cent NaCl, pH 7 6 Such solutions are stable for several hours at room temperature

Enzyme solution Urokinase preparations to be assayed are dissolved in 0 1 M phosphate buffer, pH 7.6, containing 1 per cent gelatin and 1:10,000 merthiolate to yield solutions containing on the order of 1000-2000 C.T.A (Committee on Thrombolytic Agents) or Ploug units/ml. Concentrated urokinase preparations in this buffer are stable for long periods in the deep freeze.

* May be obtained from Cyclo Chemical Corp, Los Angeles, Calif

become limiting. Furthermore, since profibrinolysin is also present in and added with the thrombin, the addition of thrombin via the rod technic introduces appreciable errors under conditions where lysis times are so short as to render the profibrinolysin content of the system limiting.

3. No fibrinogen or thrombin preparation should be employed which produces a clot having a lysis time longer than 3 minutes in the presence of a concentrated preparation of activator (100 units per ml or more of urokinase). In addition no fibrinogen or thrombin preparation should be employed which produces a clot with a control lysis time (no added activator) of less than 48 hours.

4. Other unit definitions of urokinase are available in the literature. By using the bear assay of Kjeldgaard and Ploug[3] with urokinase prepared by the method of Celander and Guest, and by assaying the Leo preparations *by the one-stage method, the one-stage unit described above has been found* to equal approximately 10 of the Leo units Comparison of the unitage of normal human urine with the values obtained by von Kaulla[4] indicates that 10 of the one-stage units are about equal to one of the von Kaulla units.

5. Slight modifications of the methods described may be used for the assay of activators of profibrinolysin other than urokinase.

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[3] Kjeldgaard, N O, and Ploug J Urokinase, an activator of plasminogen from human urine Biochem et Biophys Acta *24* 283, 1957.

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The final calculation is as follows:

$$\frac{\text{O.D. (assayed solution)} \times 16.5 \text{ (dilution factor)}}{\text{O.D. (1 } \mu\text{mole/ml. } CH_3OH\text{)}} \times 2 \text{ (30-minute reading)}$$

or 4 (15-minute reading) = μmoles CH_3OH liberated per ml enzyme solution per hour

The final result is the average of the calculation obtained from the 15- and 30-minute reading

Advantages. The procedure described is sensitive, rapid and simple, and has several advantages over other methods used for measuring the hydrolysis of synthetic substrates: (a) multiple determinations can be run concurrently; (b) blank values are very low even with plasma, (c) each assay allows for a check on the kinetics of the reaction as well as providing two separate determinations; (d) unlike colorimetric methods involving the Hestrin ester reaction, one measures the formation of end product rather than disappearance of substrate, an attribute which allows for the more accurate assay of small amounts of enzyme activity

In addition to being a very sensitive substrate for plasmin and urokinase, AcLMe does not undergo significant spontaneous hydrolysis at pH 7 6 for long periods of time—thus, by prolonging the incubation time (and, if necessary, by varying the amount of enzyme solution to be tested), the method can be modified readily to measure reasonably small amounts of enzyme activity

Precautions and Sources of Error Care should be used in the preparation, storage and handling of the chromotropic acid reagent, and fresh solutions of sodium sulfite should be prepared daily EDTA in high concentrations should be avoided since it gives a false positive color reaction

The technic for urokinase assay has not been applied to the assay of urokinase in urine; the method as described is not sensitive enough to assay the low concentrations normally present in urine nor has it been determined that urine may not contain other enzymes capable of splitting AcLMe.

Finally, since multiple enzymes may hydrolyze the same synthetic substrate, care should be used in the interpretation of such esterase activity when measured in enzyme mixtures or in complex biological fluids Appropriate controls may eliminate some of these problems.

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Trichloracetic acid: Fifteen per cent in water.

Sodium sulfite: Ten per cent in water made up fresh daily in small amounts.

Potassium permanganate: Two per cent in water.

Chromotropic acid reagent: To 200 ml cold distilled water in a liter volumetric flask, add 100 ml. 2 per cent chromotropic acid (4, 5-dihydroxy-2, 7-naphthalenedisulfonic acid, disodium salt (Eastman)). Place in an ice bath and slowly add 600 ml. cold concentrated H_2SO_4 in 50 ml. aliquots, allowing 20 minutes between each addition. Allow to stand overnight in cold, bring up to room temperature and add water to 1 liter. Filter through a medium-pore, sintered glass funnel. The solution is stable indefinitely under refrigeration; however, it should be discarded if it turns brown on standing. Since this is a strong acid, it is desirable to use an automatic pipette-solution container (e g., Schucopolypette with 4 ml. interval) for storage and delivery.

Procedure

1. To 3 ml substrate in a test tube, add 0.3 ml enzyme solution to be assayed and place in a water bath at 37°C. After 1 minute, remove 1 ml aliquot (zero specimen to serve as blank) into a tube containing 0 5 ml. 15 per cent TCA, and start timer. Remove additional 1 ml. aliquots at 15 and 30 minutes into separate tubes containing 0.5 ml. 15 per cent TCA.

2. To 0.5 ml of each specimen to be tested, add 0 1 ml. $KMnO_4$. Wait 1 minute.

3. Add 0.1 ml sodium sulfite reagent to completely decolorize all excess $KMnO_4$.

4. Add 4.0 ml. chromotropic acid reagent, mix well and place in a boiling water bath for 15 minutes.

5. Remove specimens from the boiling bath and, after a few minutes for cooling (color stable), read in a Beckmann spectrophotometer at 580 mμ against the zero specimen as a blank (note blanks will be higher in the presence of EDTA).

Calculations

Since the results are expressed as μmoles CH_3OH liberated per hour, a standard curve is run with methyl alcohol. These standards should be made up in 0.066 M phosphate buffer containing 5 per cent TCA, but it is recommended that the original methyl alcohol reagent be weighed out because of its low specific density. In our laboratory, 0.5 ml of a μmole/ml. solution treated from step 2 in the procedure, gives an optical density reading of 0 200. However, because of variations, it is advised that a standard be run with each assay.

ture for 30 minutes at 37°C. and the optical density of the supernat solution after addition of trichloracetic acid is determined. Units inhibitor are calculated from the units of fibrinolysin inhibited.

The separation of the two inhibitor activities in the assays depe upon (1) the relative rates of the inactivation processes and (2) the lo concentration of the immediate inhibitor in plasma when compared to concentration of the slow inhibitor.

Reagents

Casein: Hammersten casein supplied by Amend Chemical Co., N York City, is further purified as follows Suspend 50 Gm. of the dry pow in 1 liter of distilled water and stir vigorously with a mechanical stir Centrifuge for 10 minutes at 1500 rpm Decant and discard supernat portion Add 200 ml. distilled water to a 250 ml centrifuge tube a resuspend precipitate by vigorous stirring Repeat centrifugation and d card the supernatant. Add 200 ml. of 95 per cent ethanol to each tube a stir until a slurry is obtained Pour the slurry into a Buchner funnel a filter off alcohol. While in the funnel, wash the precipitate twice with per cent ethanol and 3 times with anhydrous ether Allow ether to ev orate and grind powder in mortar. The casein is of satisfactory purity wh 2 0 ml of a 2 per cent solution, precipitated by 3 0 ml of 10 per c trichloracetic acid, gives a supernatant fluid with an optical density greater than 0.080 at 280 mμ

The 4 per cent solution of casein is buffered at pH 7 4 Suspend 4 G of casein in about 70 ml. of borate-saline buffer, pH 7 4, with vigor stirring. Add 0 25 ml N NaOH per Gm of casein to convert it to t sodium salt. Continue vigorous stirring for 30 minutes or more to compl solution. Adjust pH 7.4, using glass electrode, by slow addition of N NaO Bring volume to 100 ml by addition of borate-saline buffer Remove a insoluble material by centrifugation Store solution in refrigerator Prepa tion is usable for 1 week.

Borate-saline buffer: Prepare buffer by mixing 0 05 M sodium bor (19 108 Gm $Na_2B_4O_7 \cdot 10H_2O$/liter distilled water) with 0 2 M boric aci salt solution (12.404 Gm. H_3BO_3 and 2.925 Gm NaCl/liter distilled wate until pH 7.4 is obtained with a glass electrode Approximately 1 2 parts the borate solution to 8 8 parts of the boric acid-salt solution are require

Fibrinolysin (plasmin). Use the human enzyme prepared by Kline method (p. 268). The preparation should have a specific activity of 80 10^{-3} or more caseinolytic units per mg Solution of fibrinolysin is made 0 0025 M HCl and diluted to the desired strength with borate-saline buff

Methylamine. A 5 M solution is prepared by dissolving C P methy amine hydrochloride in borate-saline buffer, adjusting the pH to 7 4 wi

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12. Assay of the Fast Acting and Slow Acting Inhibitors of Fibrinolysin (Plasmin) in Plasma (Method of Norman and Hill)

Adapted by M. M. GUEST

Object: The assays are designed to measure the activities inhibitors of fibrinolysin (plasmin) in human plasma Two inhibitors are measured. The first is the "immediate inhibitor" which Norman designates as α-2 antiplasmin (α-2 antifibrinolysin) The second, the "slow inhibitor," is an α-1 globulin which Norman calls α-1 antiplasmin (α-1 antifibrinolysin) The physiologic significance of the difference in the rate of inactivation by the two inhibitors is unknown.

Principle: Norman has reported that in human plasma two inhibitors of fibrinolysin (plasmin) can be separated by electrophoresis. One of the inhibitors migrates as an α-1 globulin It is heat labile; it combines nondissociably with fibrinolysin at a rate which depends on the temperature. This is the "slow inhibitor"

The "immediate inhibitor" migrates in an electrophoretic field as an α-2 globulin It is more heat stable than the slow inhibitor and is more stable to extremes of pH

The assay of the immediate inhibitor, as described by Norman and Hill, is a one-stage assay in which fibrinolysin is incubated at 37°C for 30 minutes with plasma and casein After precipitation of the residual casein with trichoracetic acid, the concentration of the products of digestion of casein are measured by optical density. Inhibition of digestion of casein is related to the immediate inhibitor activity of the plasma tested Units of inhibitor are given as the units of fibrinolysin inhibited or as percentage inhibition

The assay of the slow inhibitor is a two-stage assay In the first stage, fibrinolysin and diluted plasma in the presence of methylamine are incubated at 25°C for 180 minutes The methylamine inhibits spontaneous inactivation of fibrinolysin. In the second stage, casein is incubated with the mix-

which the ordinates are the same as in the first curve but the abscissae are expressed in units of fibrinolysin This is the standard reference curve for a given preparation of fibrinolysin and casein. If different reagents are used, a new standard curve should be prepared.

Standard fibrinolysin curve with methylamine present: The assay described above is repeated but the digestion is carried out with 0 1 M methylamine present in the digestion system. The values obtained are used to plot a second standard fibrinolysin curve with the abscissae in units of fibrinolysin (1 mg fibrinolysin equals 83 4 × 10^{-3} units) and the ordinates representing the optical densities obtained from the digestion of casein by the various concentrations of fibrinolysin in the presence of 0.1 M methylamine. This curve is used to determine the fibrinolysin concentration which is available for inhibition by the slow inhibitor.

Immediate inhibitor assay: Determinations in duplicate are recommended. Pipette 0 5 ml aliquots of diluted plasma into 16 × 100 mm. test tubes. Add 1.0 ml. of 4 per cent casein, pH 7.4 (if immediate inhibitor activity of plasma is low, 1 0 ml aliquots of plasma may be used with 0 5 ml of 8 per cent casein) Place tubes in 37°C bath and add 0 5 ml. of 0 0025 M HCl containing 0 2 mg of fibrinolysin (approximately 80 × 10^{-3} units per mg.) Start timing when fibrinolysin is added. Control tubes contain casein, fibrinolysin and buffer instead of plasma. Stop reactions after 30 minutes of incubation by adding 0 5 ml of 15 per cent trichloracetic acid An additional 2 5 ml of 15 per cent trichloracetic acid is added 5 to 10 minutes later Blanks for each plasma tested are prepared by adding the trichloracetic acid before the fibrinolysin is added Allow tubes to remain overnight at 4°C Separate precipitates by centrifugation at 2700 rpm for 1 hour. Read optical density of each supernatant solution in Beckman DU spectrophotometer at 280 mμ The standard curve can be used to convert the optical density readings to units of fibrinolysin.

Calculation

Immediate inhibitor. From the standard curve, convert the optical density readings to units of fibrinolysin Subtract units of fibrinolysin obtained following incubation with plasma from units of fibrinolysin obtained in control tubes to obtain units of fibrinolysin inhibited Calculate units of immediate inhibitor per ml of plasma from the dilution of plasma in the casein digestion mixture. Thus, if the dilution of plasma is 1 to 2 and 0 5 ml of the dilution is used in a 2 0 ml digestion system, the total dilution is 2 × 4 = 8

Since it is difficult to adjust the fibrinolysin concentration to the same value when assays are performed at different times and the number of units of fibrinolysin inhibited depends on the amount of fibrinolysin avail-

N HCl or N NaOH, and adjusting the volume with the borate-saline buffer.

Human plasma: Ten ml. of blood are obtained by venepuncture and immediately transferred to tubes containing the dried residue of 1.2 ml. of 1 per cent disodium versenate (disodium ethylene diamine-tetracetate). The tubes are corked and shaken vigorously. Cells are separated from plasma by centrifugation at 2500 rpm for 10 minutes. Plasma may be stored at 4°C. for no more than 48 hours before use. A dilution of 1 part plasma to 1 part borate-saline buffer (1 to 2) is ordinarily used in the immediate inhibitor assay and dilutions of 1 part plasma to 4 parts buffer (1 to 5) or 1 part plasma to 9 parts buffer (1 to 10) are used in the slow inhibitor assay.

Procedure

Standard fibrinolysin curve: Sufficient fibrinolysin (human plasmin, Kline), having an approximate specific activity of 80×10^{-3} units per mg. is dissolved in 0.0025 M HCl to give a concentration of 40×10^{-3} units per ml. Dilutions of this solution are made with 0.0025 M HCl. Concentrations of 30×10^{-3}, 20×10^{-3}, and 10×10^{-3} units per ml. give an adequate range for preparation of the standard curve. The original concentration and each dilution should have a sufficient volume to permit 4 aliquots of 0 5 ml to be pipetted from each solution. Five-tenths ml. aliquots are added to each of two 16 × 100 mm. test tubes, each containing 1 ml. of 4 per cent casein in borate-saline buffer at pH 7.4, plus 0.5 ml. borate-saline buffer at pH 7.4 Two blanks are prepared for each dilution with casein and borate-saline buffer to which 0.5 ml. of 15 per cent trichloracetic acid is added before adding the aliquots from the fibrinolysin solution. The test tubes are incubated for 30 minutes in a waterbath at 37°C At the completion of the incubation, 0.5 ml of 15 per cent trichloracetic acid is added to each of the digestion tubes About 10 minutes later an additional 2 5 ml of 15 per cent trichloracetic acid is added to each of the tubes (including blanks). The tubes are allowed to stand overnight at 4°C. The precipitates are centrifuged for 1 hour at 2700 r p m. The optical density of each supernatant solution is then read in a Beckman DU spectrophotometer at 280 mμ

The optical densities are plotted as the ordinates against the dilutions of the fibrinolysin as the abscissae One proteolytic unit is the activity which gives rise, under the conditions of the test, to an increase in 1 unit of optical density at 280 mμ per minute of digestion The specific activity of the fibrinolysin preparation assayed is obtained by drawing a straight line tangent to the lower portion of the curve The slope divided by 30 times the dilution is the proteolytic activity per ml of the undiluted, initial solution of fibrinolysin From this information a new curve is plotted in

cent trichloracetic acid is added to all tubes except the blanks. Five to 10 minutes later, 2.5 ml. of 15 per cent trichloracetic acid is added to all tubes. After standing overnight, the precipitates are separated by centrifugation for 1 hour at 2700 r.p m The optical densities of the supernatant solutions are read at 280 mμ in the Beckman Du spectrophotometer.

Calculations

Slow inhibitor: Convert optical density to fibrinolysin activity in units by reference to the standard curve. Subtract units of activity obtained with plasma from units of activity obtained in control assay to obtain units of fibrinolysin inhibited. To calculate units of fibrinolysin inhibited, multiply by dilution of plasma in the casein digest mixture. Thus if 0.5 ml. of a 1 to 10 dilution of plasma is used, the dilution will be 10 × 2 (0.5 plasma in a 1 ml incubation system) × 10 (0 2 ml. of incubation mixture in a 2 ml. digestion system) = 200 Calculated units of slow inhibitor, according to Norman and Hill, are not influenced by the fibrinolysin concentration if sufficient fibrinolysin is used. Therefore it is not necessary, as with the immediate inhibitor, to compare determinations which are made at different times on a percentage inhibition basis

Precautions and Notes: 1. Casein of high purity is required 2. A new standard curve for converting units of optical density to units of fibrinolysin should be prepared for each different preparation of casein or fibrinolysin 3. The plasma should not be allowed to stand more than 48 hours before being assayed During this period it must be stored at 4°C 4 Units of the immediate inhibitor vary with the amount of fibrinolysin used in the assay and therefore determinations on different days must be compared as percentage inhibition 5 Since the activity of the immediate inhibitor is only about 5 per cent of that of the slow inhibitor, it is not essential to subtract the activity of the immediate inhibitor from that of the slow inhibitor.

Normal Values: In a group of 18, presumably normal males and females, Norman and Hill found the immediate inhibitor to vary between 49 5 and 61.2 per cent inhibition with a mean of 56 4 per cent inhibition In the same group the slow inhibitor varied between 700 and 990 units/ml plasma with a mean of 850 units/ml plasma

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Norman, P S, and Hill, B M. Studies of the plasmin system. III Physical properties of the two plasmin inhibitors in plasma J Exper Med *108* 639, 1958

able for inhibition, Norman and Hill recommend that assays for immediate inhibitor, carried out on different days, be converted to percentage inhibition, if the levels of inhibition are to be compared. However, the relationship between the amount of inhibitor and the percentage inhibition is not linear and therefore approximation of the recommended concentration of fibrinolysin is advisable.

Procedure

Slow inhibitor assay: Diluted plasma, methylamine and fibrinolysin are incubated for 18 minutes at 25°C. in the first stage. During the second stage, the residual fibrinolysin which has not been inactivated by the slow inhibitor is allowed to digest casein The optical densities produced by the soluble products of casein digestion are measured at 280 mμ. A control sample is carried through the same procedures. In the control system, borate-saline buffer replaces plasma The difference in fibrinolytic activity between the system without plasma (control) and the system with plasma is a measure of the slow inhibition.

To perform the assay, the plasmas are diluted with borate-saline buffer. Usually a 1 to 5 or a 1 to 10 dilution gives a satisfactory end point. The incubation mixtures are prepared in 16 × 100 mm. test tubes. One tube is required for each plasma. To 0.5 ml of the diluted plasma, add 0.4 ml. of 5 M methylamine, buffered at pH 7 4, and place the tube in a water bath at 25°C. Then add 0.1 ml. of 0.0025 M HCl containing 1 mg. of fibrinolysin (approximately 80 × 10^{-3} units per mg.) and incubate for 180 minutes. For each series of plasmas to be assayed at any one time, prepare at least one control tube containing borate-saline buffer in place of the plasma. This tube, containing 0 5 ml of borate-saline buffer, 0.4 ml 5 M methylamine and 0 1 ml. of the fibrinolysin solution, should also be incubated for 180 minutes at 25°C.

Duplicate samples are prepared for the second-stage incubation. Duplicate blanks, in which the casein is precipitated by trichloracetic acid before digestion, are also prepared for each plasma sample and for each control. The second-stage incubation mixture consists of a 0 2 ml aliquot from the first-stage incubation samples (plasma and control), 0.8 ml. of borate-saline buffer and 1 0 ml. of 4 per cent casein The aliquot is added to the casein and buffer after prewarming to 37°C. and at the completion of the 180-minute first-stage incubation Duplicate blanks are also prepared for each plasma system and for each control. Each blank contains a 0.2 ml aliquot of a first-stage incubation mixture (plasma or control), 0.8 ml of borate-saline buffer and 1.0 ml. of 4 per cent casein In addition, 0.5 ml. of 15 per cent trichloracetic acid is pipetted into each blank before incubation at 37°C. At the end of the 30-minute digestion period, 0.5 ml. of 15 per

to stir the fibrinogen-fibrinolysin mixture Clotting occurs within 15 seconds. The lytic end point is reached when the contents of the tube flow freely without humping as the tube is slowly tipped so that its open end is about 10° below the horizontal. The tube is examined in this way at frequent intervals, but is returned to the 28°C. water bath after each examination

To assay a given plasma for its antifibrinolysin content, 0.1 ml. of the diluted citrated or oxalated plasma (1 to 5 or 1 to 10 dilution) is added to 0 1 ml. of a solution containing 0 4 unit of fibrinolysin The tube is incubated for 30 minutes in a water bath at 28°C. At the completion of the 30-minute incubation, 0 2 ml. of 0 2 per cent fibrinogen is added to the tube coincidently with the starting of the stop watch and thrombin is added by stirring rod The tube is returned to the 28°C. water bath and examined at frequent intervals for evidence of lysis. The end point is the complete dissolution of the clot as described in the standardization of the fibrinolysin solution.

Calculations. Five units of antifibrinolysin, within 30 minutes at 28°C. and pH 7.2, will reduce the activity of 1 unit of fibrinolysin to 0 5 unit To obtain a standard curve for a given species from which the units of antifibrinolysin can be estimated, it is necessary to plot a series of pooled plasma dilutions against the dissolving time of the standard clot The 210-second lysis time should be determined accurately since this corresponds to an inactivation of one-half of the fibrinolysin added to the system. This point on the curve corresponds to 5 units of antifibrinolysin according to the definition of antifibrinolysin units, given above. Having found this point on the curve, the ordinate can then be divided into fractions and multiples of 5 units The antifibrinolysin assay curves for the pooled plasmas of a number of species are available in the original paper of Guest et al.[1]

When the lysis time of the standardized fibrin clot containing diluted plasma and fibrinolysin has been determined as described above, the number of units of antifibrinolysin are read on the ordinate of the graph for the particular species However, since the units have been established for a 1 ml clot, and a 0 4 ml clot is ordinarily used (to conserve reagents), a correction must be made Furthermore, since the plasma is diluted in preparation for the assay, an additional correction must be made Thus, if 1 ml of anticoagulant were added to 7 ml of blood, 6 units of antifibrinolysin were read on the graph, the dilution of the plasma was made by adding 9 parts of saline to 1 part of blood, and the hematocrit was 40, the calculation would be·

$$\underline{A} \times \underline{B} \times \underline{C} \times \underline{D} = \underline{Ua}$$

$$\frac{(1\ 00-0\ 40)\ 8}{((1\ 00-0\ 40)\ 8)-1} \times \frac{10}{1} \times 4 \times 6 = 273$$

13. Assay for Antifibrinolysin (Antiplasmin)

M. M. GUEST, B. M. DALY, A. G. WARE and W. H. SEEGERS

Object. The assay[1] is designed to measure the total antifibrinolytic activities of plasma.

Principle: The enzyme, fibrinolysin (plasmin), is inactivated when incubated with plasma. Inactivation is presumed to result primarily through a complexing of fibrinolysin with specific antifibrinolysins. Norman[2] and others have demonstrated that both an immediate inhibitor, an α-2 globulin, and a slow acting inhibitor, an α-1 globulin, are present in plasma The method described here is a measure of the activity of both inhibitors plus any non-specific inhibition which may occur.

Reagents: Imidazole buffer, pH 7 25; Thrombin topical, Parke, Davis & Co.; fibrinolysin (bovine) prepared by the method of Loomis, George and Ryder; fibrinogen (bovine) prepared by the freeze-thaw technic,[3] or another source of relatively pure fibrinogen.

Procedure: To plasma, that has been obtained from blood to which either sufficient potassium oxalate or sodium citrate to prevent clotting was added at the time of drawing, a small amount of thrombin in the dry form is added and the fibrin is removed by winding it onto a glass stirring rod Either the plasma or the serum may be stored at −20°C. before performing the assay Just prior to the assay, suitable dilutions of the plasma are made with a solution containing 1 part of 0.9 per cent NaCl and 2 parts of imidazole buffer at pH 7 2. Usually a 1 to 5 or a 1 to 10 dilution will bring the time of lysis into a satisfactory range

A solution of fibrinolysin is prepared in imidazole buffer, pH 7 2, which when tested with 0.1 per cent fibrin clot, formed by the addition of 0.1 ml. of the fibrinolysin solution, 0 1 ml of 0 9 per cent saline and 0 2 ml. of a 0.2 per cent fibrinogen solution, brings about complete lysis at 28°C. in 120 ± 5 seconds When lysis of the 0 4 ml. clot occurs in 120 seconds, the clot contains 0.4 unit of fibrinolysin since 1 unit of fibrinolysin is defined as the amount which will completely lyse 1 ml of a 0 1 per cent fibrin clot at 28°C. in 120 seconds in an isotonic saline solution buffered with imidazole at pH 7 2

The test tubes used in the assay are 50 mm × 8 mm I D The fibrinolysin and saline solutions are pipetted into the tube and a stop watch is started with the addition of the fibrinogen solution The fibrinogen is clotted by the addition of thrombin on the end of a stirring rod which is dipped to a depth of 5 mm in a solution of thrombin (saline-glycerol mixture containing 1433 N I H units of thrombin per ml) before it is used

14. Preparation of Antifibrinolysin (Antiplasmin) (Method of E. C. Loomis)

Adapted by M. M. GUEST

Object: The albumin fraction obtained by the method described[1] will inhibit fibrinolysis in vitro and may be used for the inhibition of fibrinolysis in experimental animals.

Principle Bovine, equine or human antifibrinolysins are precipitated at 5°C. by 70 per cent saturation with $(NH_4)_2SO_4$.

Reagents and Apparatus

$(NH_4)_2SO_4$, H_2SO_4 and NaOH (C P.).

Visking "No Jax" casing, 29/32 inch, refrigerated centrifuge; glassware.

Procedure Serum is prepared as described in the method of Loomis for the preparation of fibrinolysin.

One liter of serum is cooled to 5°C and brought to 50 per cent saturation by adding an equal volume of cold, saturated $(NH_4)_2SO_4$ The precipitate is removed by centrifugation in the cold. To each 100 ml. of the supernatant fluid at 5°C., 13 5 Gm. of $(NH_4)_2SO_4$ is added with stirring; this brings the degree of saturation to approximately 70 per cent After centrifugation in the cold, the supernatant fluid is removed and discarded. The precipitate is dissolved in 100 ml. of distilled water and dialyzed for 18 to 20 hours in Visking "No Jax" casings against cold running tap or deionized water

The contents of the dialysis bag are again brought to 50 per cent of saturation at 5°C by the addition of an equal volume of saturated $(NH_4)_2SO_4$ and adjusted to pH 3.75 with N H_2SO_4. The precipitate is removed by centrifugation in the cold and the supernatant fluid is adjusted to pH 7.0 with N NaOH The preparation is dialyzed as above. The dialyzed fluid may be shell-frozen, lyophilized, and the dry powder stored in a desiccator

Precaution and Notes 1. Temperature control as indicated appears to be important 2 The pH during the acidic precipitation must be between pH 3 5 and 4 0 3 The antifibrinolysin prepared in this way may be assayed by the method of Guest, Ware, Daly and Seegers,[2] a solution of the antifibrinolysin in 0 9 per cent NaCl is substituted for serum. 4 The best preparations are about 85 per cent pure on electrophoretic analysis 5. This inhibitor of fibrinolysis is an albumin, other inhibitors are found in the globulin fractions.

where

A = correction for dilution due to anticoagulant;
B = dilution of plasma with saline;
C = correction for 1 ml. clot.

This correction is arrived at as follows: *Since 0 1 ml. of diluted plasma is used to inactivate a certain fraction of the fibrinolysin in a 0 4 ml. clot,* 0.25 ml. of plasma would be required to inactivate the same fraction of fibrinolysin in a 1.0 ml. clot and 1 ml. of plasma would contain 4 times as much antifibrinolytic activity as was obtained in the assay with a 0.4 ml. clot.

D = units of antifibrinolysin which are read from the standard curve for the species being studied.

Ua = units of antifibrinolysin per ml plasma.

Normal Range of Values: Man. 55 to 100 units; dog: 40-80 units.

Precautions and Notes: 1 The assay is based on the inhibition of the enzyme, fibrinolysin. If inhibition of trypsin is used in the procedure, the results will be obtained in antitrypsin units and not antifibrinolysin units 2. If a lysis time other than 120 ± 5 seconds is used for the standard *fibrinolysin preparation (without added antifibrinolysin) in the* assay, a new standard curve relating lysis times to antifibrinolysin units must be prepared. 3 It is convenient to prepare the fibrinolysin solution with a higher enzyme concentration than that which would be expected to give 120 seconds in the assay and make dilutions of this solution to bring its *lysis time, when tested without added plasma, to 120 ± 5 seconds.* 4. The stability of the standardized bovine fibrinolysin solution is enhanced by keeping it in an ice bath between assays, performed within a 2- to 3-hour period. 5. Dog plasma normally contains less antifibrinolytic activity than is found in the plasma of man Therefore, canine plasma dilutions of 1.2 or *1.4 usually suffice* 6 *In the original paper of Guest et al.,* correction C in *the calculation of antifibrinolysin unitage was inadvertently omitted*

References

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[2] Norman, P S, and Hill, B M Studies of the plasmin system III Physical properties of the two plasmin inhibitors in plasma J Exper Med 108· 639, 1958.

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2. *Platelet-rich plasma.* Blood collected with siliconized apparatus into EDTA solution and centrifuged at 800 rpm for 10 mins in an International "Clinical" centrifuge.

3. *Platelet-poor plasma.* Plasma collected as above, but centrifuged at 16,000 rpm for 20 minutes (Lourdes Refrigerated Centrifuge, 9 RA head.)

4. *Casein substrate.* Five Gm. casein (Hammersten quality, Nutritional Biochemical Co., Cleveland, Ohio.) is suspended in 100 ml of phosphate saline buffer at pH 7.6 and the pH adjusted to 7.6. The solution is placed in a boiling water bath for 15 minutes, cooled, filtered and the pH readjusted to pH 7.6. The final casein solution is opalescent

5. *Folin-Ciocalteu reagent* (Fisher Laboratory reagent).

6. *Phosphate-saline buffer* refers to 0 1 M phosphate in 0.15 M sodium chloride at pH 7 6.

Assay In each of eight tubes is measured 0.5 ml plasmin solution (3 5–4 0 cas. U./ml). To two control tubes is added 0 2 ml. 0.1 M phosphate saline buffer at pH 7 6. Platelet-rich plasma aliquots (0 2 ml), respectively undiluted and diluted 1:2 and 1:4 with phosphate saline buffer, are added to half the remaining tubes and similarly diluted aliquots of platelet-poor plasma are added to the remainder After incubation at room temperature for 15 minutes, the tubes are transferred to a 37°C water bath. Two and three-tenths ml 0.1 M phosphate saline buffer and 2 ml 5 per cent casein solution are added to each tube After 2 and 62 minutes incubation, 2 ml of each digestion mixture is added to 2 ml 10 per cent trichloracetic acid After centrifugation the supernatant is assayed for "tryosine" either by absorption at 280 mμ or by the Folin Ciocalteu reaction as follows 1 ml supernatant, 1 5 ml 5 per cent trichloracetic acid, 5 ml 0.5 N NaOH and 1 5 ml Folin-Ciocalteau reagent (diluted 1:3) are mixed thoroughly After 20 minutes the absorption of the 62-minute sample is read, using the 2-minute sample as a blank, in a Beckman spectrophotometer at 650 mμ

Results for each individual assay are expressed as percentage inhibition of the control plasma solution and plotted as shown in figure 1, with percentage sample inhibition on the ordinate and the log of the plasma concentration on the abscissa A linear plot result and individual sample assay lines are approximately parallel to each other Platelet inhibitory activity may be expressed as a percentage of plasma inhibitory activity and is calculated by dividing inhibitory activity of platelet-rich plasma by that of the platelet-poor plasma Alternatively, platelet inhibitory activity can be converted to an absolute figure, but because plasma contains multiple inhibi-

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15. Platelet Antiplasmin

N. ALKJAERSIG

Object of the Test: To quantitate the total platelet antiplasmin content and its variation in disease

Principle. Antiplasmin contained in or adsorbed to platelets exerts at least two actions of physiologic interest: first, platelet antiplasmin may supplement plasma antiplasmin, and second, because of the incorporation of platelets into clots, it may influence the susceptibility of clots to subsequent lysis. Consequently, measurement of this parameter may involve assays of divergent type, the one providing a biochemical expression of platelet inhibitory action against plasmin and the other a physiologic one reflecting the inhibitory action of platelets on thrombolytic phenomena. This latter action may be quantitated by determining the inhibitory action of platelets incorporated into I^{131}-labeled plasma clots tested against a standard plasminogen activator concentration [1] However, since the direct biochemical determination of platelet inhibitory action against plasmin is the more generally useful procedure and technically the easier, it has been chosen for description.

The test employs whole platelets suspended in plasma, as in this way loss of platelets or their disruption are minimized. The inhibitory actions of platelet-rich and platelet-poor plasma are tested against *activator free* plasmin[2] and the value for platelet antiplasmin is obtained by subtraction. However, if desired, isolated platelets suspended in buffer may also be employed.

Reagents

1. *Plasmin.* Human plasmin prepared by the Kline procedure from Cohn fraction III and activated in 50 per cent glycerol[2] or by streptokinase and subsequently rendered activator-free by precipitation in 1 M NaCl at pH 2.0.[3]

CHAPTER XI

ANTICOAGULANTS IN BLOOD, PLASMA AND SERUM

1. Detection of Endogenous Circulating Anticoagulants

C. L. CONLEY and D. P. JACKSON

Abnormal anticoagulant activity in blood is demonstrated by the clot retarding effect of the blood or plasma on a normal coagulation system. Methods available are of varying specificity and sensitivity. The method most suitable for the detection of one type of anticoagulant may be insensitive to another, therefore, no single method can be selected as superior.

NON-SPECIFIC METHODS

1. *Demonstration of Abnormal Anticoagulant Activity in Plasma (Adapted from Conley, Hartmann and Morse[3])*

Principle Platelet-poor plasma from normal blood invariably clots in glass tubes at 37°C and does not significantly prolong the clotting time of normal blood to which it is added If abnormal anticoagulant activity is present, platelet-poor plasma does not clot in glass tubes and may retard coagulation of normal blood with which it is mixed

Technic Needles, syringes, test tubes and pipettes are treated with silicone according to the method of Jaques (see page 4) A non-traumatic venepuncture is performed on the test subject employing an 18 gauge needle, and 20 ml of blood are carefully withdrawn The blood is transferred to a silicone-coated tube in an ice bath, and is immediately centrifuged at high speed (about 20,000 g) for 10 minutes at 4°C. The upper half of the supernatant plasma, which is virtually free of cells and platelets, is carefully removed with a silicone-coated pipette and stored in an iced silicone-treated tube until used Normal plasma prepared in this way remains fluid for hours at 4°C. and plasma containing traces of anticoagulant does not clot even when transferred to glass tubes at 37°C.

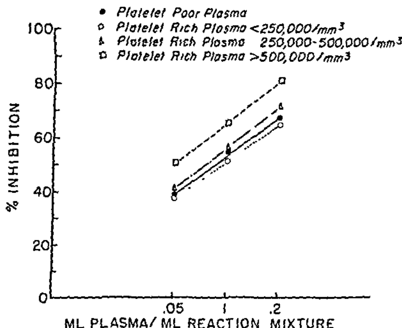


Fig. 1.—Antiplasmin assays on platelet rich and platelet poor plasma.

tors such an expression of values is to a degree arbitrary, being partially dependent upon the assay conditions employed.[3]

Normal Values and Range: Platelet inhibitory activity appears to be primarily a function of platelet count. Platelet antiplasmin values were below 3 per cent of plasma antiplasmin with platelet counts below 250,000/mm.,[3] ranged from 3-10 per cent with counts of 250,000-500,000/mm.,[3] and exceeded 10 per cent when the count was greater

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kept in an ice bath. Three test tubes are labeled respectively J (joint), S (separate) and N (normal), and are placed in the ice bath. Nine-tenths ml. of the test plasma are pipetted into each of tubes J and S; 0 1 ml. normal plasma into tube J and 1 0 ml. normal plasma into tube N. The tubes are covered with Parafilm* and the contents of tube J mixed by inversion. The tubes are transferred to a water bath at 37°C., incubated for 60 minutes and then are returned to the ice bath. After incubation, 0.1 ml. of plasma from tube N is transferred to tube S and the contents of the latter mixed by inversion

Clotting times of the jointly and separately incubated plasma mixtures are determined after recalcification From tube J two aliquots of 0.2 ml. each are transferred into separate test tubes at 25°C. Immediately, 0.2 ml. of M/40 calcium chloride are added to each tube and the clotting time measured with a stop watch In similar fashion the recalcified clotting times of plasma in tubes S and N are determined.

Interpretation. Significant prolongation of the recalcified clotting times of mixtures of test and normal plasma indicate the presence of anticoagulant activity If the clotting time of the jointly incubated mixture is longer than that of the separately incubated plasma, the anticoagulant is one which progressively inhibits one or more coagulation factors. By varying the concentration of test plasma in the mixtures, the activity of the anticoagulant can be titrated This method is very sensitive for the detection of anticoagulants inhibiting the early stage of coagulation

Precautions and sources of error. Prolongation of the clotting time of the jointly incubated plasma mixture is not considered significant unless it exceeds the coagulation time of the normal control or of the mixture of separately incubated plasmas by at least 2 minutes. An anticoagulant present in high titer, or one whose effects are not increased by incubation, may prolong the clotting times of both the jointly and separately incubated mixtures

3 *Demonstration of Abnormal Anticoagulant Activity in Plasma by Use of the Thromboplastin Generation Test (Adapted from Biggs and Douglas*[2]*)*

Principle Plasma from the test subject is incorporated in the reagents of the thromboplastin generation test. If anticoagulant activity inhibiting the early stage of coagulation is present, thromboplastin generation is delayed

Technic The thromboplastin generation test is performed in the usual way (see page 89), employing normal citrated platelet-poor plasma as substrate Reagents consist of normal platelets, normal serum and adsorbed

* Parafilm, Marathon

Fifteen scrupulously clean glass tubes (13 × 100 mm, *not* silicone-treated) are mounted in a rack so that there are five ranks of three tubes The three tubes in the first row contain no added material. In each of the three tubes of the second row is pipetted 0.2 ml. of 0.85 per cent NaCl, in the third row 0.5 ml. of 0.85 per cent NaCl; in the fourth row 0.2 ml. of platelet-poor plasma, and in the fifth row 0.5 ml. of platelet-poor plasma. With a silicone-treated needle and syringe, sufficient blood is drawn from a normal person so that 1 ml. portions can be added quickly to each of the 15 tubes. The rack is shaken to mix the contents of the tubes and is placed in a water bath at 37°C. The clotting time of each tube is recorded employing a stop watch which is started at the time that the normal blood is drawn.

Interpretation Significant prolongation of the clotting times in the tubes containing platelet-poor plasma is indicative of the presence of abnormal anticoagulant activity. When anticoagulant activity is demonstrated, its potency may be estimated by determining the smallest amount of platelet-poor plasma which will prolong the clotting time of normal blood. When normal serum is used in place of platelet-free plasma, there is no prolongation of the clotting time of whole blood. However, anticoagulants detectible in platelet-poor plasma may not be demonstrable in serum.

Precautions and sources of error. Normal platelet-poor plasma may prolong slightly the coagulation time of normal blood. In the presence of anticoagulant activity, clotting times are often two or more times as long as those of the saline controls. Caution is required in the interpretation of more equivocal data and the test should be repeated or other tests performed if the result is uncertain. This test is very sensitive for the detection of heparin and similar substances but is less sensitive than other tests for detection of inhibitors of factor VIII.

2. *Demonstration of Abnormal Anticoagulant Activity in Plasma (Adapted from Biggs and Macfarlane*[1]*)*

Principle. Citrated plasma from the test subject is mixed with normal citrated plasma and the clotting time determined after recalcification If anticoagulant activity is present in the test plasma, the clotting time of the mixture is prolonged Since certain anticoagulants progressively inhibit specific coagulation factors as a function of time, mixtures of the test and normal plasma are incubated prior to recalcification

Technic Blood is obtained from the test subject by nontraumatic venepuncture employing an 18 gauge needle and syringe Four and five-tenths ml. of blood are mixed with 0 5 ml of 3 8 per cent sodium citrate solution in a test tube. Supernatant plasma is removed with a pipette after centrifugation at 4000 g for 10 minutes Citrated plasma is prepared from normal blood in the same manner and the two specimens of plasma are

Interpretation: Significant prolongation of the recalcified clotting times of the mixtures containing jointly incubated plasma indicates the presence of an anticoagulant specifically inhibiting factor VIII.

Precautions and sources of error. Prolongation of the clotting times of both separately and jointly incubated mixtures may indicate a high-titered anticoagulant inhibiting factor VIII, a non-specific anticoagulant or low factor VIII activity in the normal plasma employed in the test. The factor VIII-deficient plasma used in the assay must have a markedly prolonged clotting time on recalcification so that corrective action of normal plasma can be detected readily.

2. *Demonstration of Anticoagulant Activity Inhibiting Specific Factors other than Factor VIII.*

Anticoagulant activity inhibiting certain other coagulation factors can be detected by modification of the method described above After separate and joint incubation of test and normal plasma, the assay is performed for the factor in question. For example, in tests for inhibitor of factor IX, plasma obtained from a patient deficient in factor IX is used instead of factor VIII-deficient plasma in the final assay procedure.

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plasma prepared as follows: In place of normal adsorbed plasma in the generating mixture, jointly and separately incubated plasmas, prepared as described in method 2, above, are employed after adsorption with aluminum hydroxide. The activities of the plasma mixtures prepared in this way are compared in the generation test.

Interpretation: Significant retardation of thromboplastin generation by jointly incubated plasma is indicative of anticoagulant activity inhibiting the early stage of coagulation.

Precautions and sources of error: Prior incubation of the test plasma with the normal plasma is necessary to make the test adequately sensitive Although anticoagulant activity in serum may be detected by simple addition of the test serum to the generating mixture, results may be difficult to interpret since normal adsorbed serum sometimes inhibits thromboplastin generation when added to the generating mixture in larger than usual amounts.

Specific Methods

1. *Demonstration of anti-Factor VIII activity in Plasma (Adapted from Biggs and Macfarlane[1] and from Lewis, Ferguson and Arends[4])*

Principle: Citrated plasma from the test subject is incubated with normal citrated plasma and the mixture is then assayed for factor VIII activity. If an inhibitor of factor VIII is present in the test plasma, the factor VIII activity of the normal plasma in the mixture is diminished

Technic The initial steps of the procedure are identical with those described in the first paragraph of "Technic" of non-specific method 2, above At this point, assay for factor VIII in the jointly and separately incubated mixtures and in the normal plasma is performed as follows: With a 1.0 ml graduated pipette, 0 9 ml of barbital buffer (sodium diethyl barbiturate 2.06 Gm , diethyl barbituric acid 2 76 Gm , sodium chloride 7.30 Gm ; water to 1000 ml , pH 7 6) is transferred into each of three test tubes labeled "J 1/10," "S 1/10" and "N 1/10" in an ice bath One-tenth ml of plasma from tube J is transferred to that labeled "J 1/10," and similarly 0.1 ml of plasma from tube S and tube N is transferred respectively to tube "S 1/10" and "N 1/10" The contents of each tube are mixed by inversion. Eight clean test tubes are placed in the ice bath so that they are aligned in groups of two One pair is labeled "J," one pair "S," one pair "N" and one pair "B" (buffer) One-tenth ml of the diluted plasma or buffer is transferred to the correspondingly labeled tube To each tube is added 0.1 ml of a known factor VIII-deficient plasma The tubes are transferred to a water bath at 25°C Immediately, 0 2 ml of M/40 calcium chloride solution are added to each tube and the clotting time measured with a stop watch

TABLE 1: *Testing of Unknown Plasma for Anticoagulant Activity Silicone Coated Tubes 38°C*

Tube no.	Normal Plasma (ml)	Unknown Plasma (ml)	0 2 M $CaCl_2$ (ml)	Clotting Time (secs)	Normal Plasma %	Unknown Plasma %
1	0 5	0 0	0 05	1,850	100	0
2	0 45	0 05	0 05	2,850	90	10
3	0 4	0 1	0 05	3,250	80	20
4	0 3	0 2	0 05	4,400	60	40
5	0 2	0 3	0 05	6,500	40	60
6	0 1	0 4	0 05	41,000	20	80
7	0 0	0 5	0 05	No Clot at 24 hrs.	0	100

expressed in terms of the proportion of unknown to normal plasma which can still delay the coagulation of the latter significantly. For example, in table 1, one part of the unknown can delay significantly the rate of clotting of nine parts of normal plasma. This proportion has been observed in certain instances to be as high as 1 in 250

Precautions and Sources of Error (1) The test must be carried out in silicone tubes, since plasma that has a clear clot-delaying activity when tested in silicone coated tubes may fail to do so in uncoated glass. If there is a great excess of anticoagulant present the clot-delaying effect may be observed even in glass tubes, while in silicone even a low content of anticoagulant will exert a clot decelerating effect (2) The normal plasma used as a substrate must be stable, when tested in a mixture such as indicated in Tube No 1 in table 1 it should have a clotting time ranging from 1200 to 2400 seconds A normal plasma that is prepared according to the directions given usually proves satisfactory (3) The plasma mixtures must have a high final plasma concentration, even after $CaCl_2$ has been added in the mixture named in table 1 the final plasma concentration in each tube is about 89 per cent

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2. Estimation of Excessive Anticoagulant Activity

L. M. TOCANTINS, R. R. HOLBURN and R. T. CARROLL

Object of the Method: The demonstration of the presence of excessive anticoagulant in unusually stable blood.

Principle: Plasma containing an excess of anticoagulant prolongs the clotting time of stable normal plasma The least amount of the unknown plasma which causes a significant increase in the clotting time of the normal plasma serves as a measure of the degree of anticoagulant activity in the unknown plasma.

Reagents and Apparatus: Siliconized 13 mm. i.d. tubes. Siliconized pipettes, graduated at 0.01 ml. *Stable plasma:* Blood is drawn from a clean venepuncture of a turgid vein using an #18 gauge needle, into a siliconized syringe containing one hundredth volume of 38 per cent sodium citrate. Immediately after withdrawal of the blood, the needle is removed and the blood and citrate well mixed by inversion. By applying gentle pressure to the plunger of the syringe, the blood is allowed to run down the wall of a siliconized test tube, to avoid bubbling and foaming The citrated blood is centrifuged at 2400 g for 50 minutes at 4°C. The upper three-fourths of the plasma is removed by means of a siliconized dropper pipette, avoiding agitation of the cellular elements Normal and unknown plasmas are prepared in the same way $CaCl_2$ 0 2 M: 2 22 grams anhydrous $CaCl_2$, C. P dissolved in 100 ml. distilled water.

Steps in Procedure. A series of seven silicone coated test tubes is set up, as shown in table 1, in a water bath at 38°C The abnormal plasma is added to the stable normal plasma and the calcium chloride added immediately after The contents are mixed by tilting the tube once and then the tube is placed back into the water bath The tubes remain undisturbed for fifteen minutes Thereafter they are checked for clotting at 100 second intervals When incipient clotting is noted the frequency of removal is increased Too frequent checking of the tubes is undersirable as it tends to shorten clotting times due to the excessive agitation of the contents

Calculation A curve of the data is plotted on semilog paper, the clotting times on the logarithmic and the contents of the mixture on arithmetic ruling A significant anticoagulant effect is considered to exist when one part of the unknown plasma is capable of delaying the clotting of no less than three parts of the normal, by a value equivalent to one-third the clotting time of an equal volume of the whole normal plasma Results are

Preparation of cephalin suspension. To 3 0 grams of acetone dried brain powder are added 75 ml. of peroxide-free ether. The mixture is allowed to extract 24 hours at 5°C. The supernatant liquid is decanted through a double thickness of filter paper and concentrated to a volume of 6 to 8 ml in a vacuum desiccator and transferred to a 50 ml. tube. Cold, absolute ethanol is added slowly to the tube until the resulting precipitate appears less concentrated at the top. This is the point of maximum precipitation. The concentration of ethanol at the point of maximum precipitation is between 77 and 80 per cent. Further addition of ethanol will cause the cephalin to assume a colloidal state and the fine particles will not settle. The precipitate is allowed to settle at 5°C until the supernatant is clear (about 30 minutes) The suspension is centrifuged 10 minutes at 2000 rpm and the supernatant discarded The precipitate is redissolved in 4 to 5 ml. of ether and the alcohol precipitation repeated A preparation six to eight times is almost free of inhibitors and is suitable for use.

After the final supernatant ethanol is discarded, the precipitate is washed into a small weighed beaker by as small a volume of acetone (10 ml maximum) as possible. The acetone is decanted and discarded when the precipitate has settled The beaker is rotated in the hand until the odor of acetone is no longer evident The powder should be white, not yellow, and yield about 1 per cent by weight of the starting material. The cephalin is immediately weighed and then suspended in a 0 85 per cent NaCl solution while scraping the sides and bottom of the beaker with a glass rod. The final concentration of the suspension should be 3 per cent A hand homogenizer is effective in making a finer dispersion of the cephalin particles The suspension should then be heated at 65°C. for ten minutes in case the preparation should be hyperactive because of thromboplastic lipoprotein contaminants The saline suspension will keep at least a month at 5°C and the cephalin powder may be stored indefinitely under ethanol without loss of potency Alternately, the cephalin may be freeze-dried (lyophilized) from the saline suspension and stored at −20°C. It is then reconstituted in an equal volume of distilled water before being used

The cephalin preparation is tested for the presence of inhibitors by noting the clot accelerating action of the suspension as it is progressively diluted With crude preparations, the initial dilutions accelerate coagulation *more than the undiluted suspension; with purified preparations, any* dilution even though small, results at once in a decrease in activity

In order to test the suspension for freedom from inhibitors the following clotting mixture is made; 0 1 ml cephalin (3 per cent), 0 1 ml citrated plasma, 0 1 ml 0 02 M $CaCl_2$ added in the order named, since if the $CaCl_2$ is added after the cephalin suspension it will flocculate it and vitiate the results A typical example of the testing follows

3. Estimation of Plasma Antithromboplastin Activity (One-Stage Method)

L. M. TOCANTINS and R. R. HOLBURN

Definitions: Antithromboplastin is a term to designate an activity, existing probably in the form of a lipoprotein in the tissues, blood, plasma and serum, directed against the formation and action of thromboplastin *Lipid antithromboplastin* is the heat stable lipid extracted from the tissues, blood, plasma or serum, which together with a plasma cofactor is capable of reducing the clot accelerating action of thromboplastin (for preparation and assay, see page 325)

Object of the Method: To estimate rapidly the degree to which a given plasma can reduce the clot accelerating activity of a cephalin suspension

Principle: Citrated plasma collected with especial precautions is incubated with a cephalin suspension for a fixed period of time. The delay in the rate of clotting caused by the incubation is compared with that caused by dilutions of the cephalin suspension tested on pooled plasma. The loss in cephalin activity is transposed into units of antithromboplastin activity.

Apparatus and Reagents Unless otherwise stated, silicone surfaces are used throughout in the collection, separation, measurement, storage and testing of plasma Plasma samples are tested on the same day of collection; frozen specimens are not used since, after thawing, they become hypercoagulable. Storage of plasma in glass tubes is likewise avoided, since glass, like clay, asbestos and similar surfaces reduces, and eventually eliminates, depending on the extent and duration of contact, most of the activity

1. Cephalin. Suspensions of cephalin are used as sources of thromboplastin instead of saline tissue extracts because: (a) Antithromboplastin activity seems to be directed against the cephalin moiety of the thromboplastin lipoprotein (anticephalin activity) (b) The great lability of saline tissue extracts as concerns thromboplastin potency, even after addition of antioxidants and other protective agents, makes them unreliable for these assays The spontaneous decrease in activity on simple standing is sometimes considerable and variable among different preparations. (c) The stability of cephalin preparations which makes it possible to standardize their activity with assurance of only minor changes in potency over several days. (d) As slow activators of prothrombin conversion, cephalin suspensions are more vulnerable to the action of inhibitors, even when these are in low concentration in the clotting mixture (e) Bacterial contamination of its suspensions is uncommon, while in saline tissue extracts, bacteria grow rapidly.

Tube No	1% Cephalin Suspension	0 85% NaCl (ml)	Plasma (ml.)	0 2 M $CaCl_2$ (ml)	Units of Cephalin Activ in Mixture	Clotting Time (secs)
1	0 1	0	0 5	0 05	180	315
2	0 075	0 025	0 5	0 05	135	370
3	0 05	0 05	0 5	0 05	90	460
4	0 025	0 075	0 5	0 05	45	660
5	0 016	0 084	0 5	0 05	30	805
6	0 008	0 092	0 5	0 05	15	1175
7	0	0 1	0 5	0 05	0	1180

In the table above the cephalin activity is expressed as units added per *1 ml* plasma; this facilitates in converting the antithromboplastin activities from *0 5 ml. (amount* used in the clotting mixture) to 1 ml. (the amount used in reporting the results) The results, clotting times as ordinates and units of cephalin activity as abscissa, plotted in log/log paper yield a straight line as shown in figure 1 The clotting times of the unincubated and the incubated plasmas are then located on the line and the correspond-

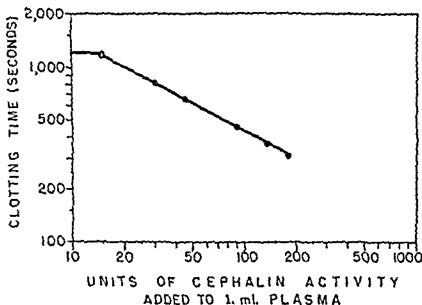


FIG 1 —Standard curve for one stage estimation of cephalin activity—pooled normal plasma

Tube No.	3% Cephalin Suspension (ml)	0 85% NaCl (ml)	Plasma (ml.)	0.02 M $CaCl_2$ (ml)	Clotting Time (secs)	
					2 × ppted. Cephalin	8 × ppted Cephalin
1	0.1	—	0 1	0 1	180	135
2	0 05	0 05	0 1	0 1	150	150
3	0 03	0 07	0 1	0 1	140	166
4	0 015	0 085	0 1	0 1	135	198
5	0 01	0 09	0 1	0 1	140	220

The 2x precipitated cephalin preparation is not suitable for assay of antithromboplastin.

2. Stable plasma. Blood from a neat, fast venepuncture from which the first few milliters have been discarded is drawn into a silicone-coated syringe containing an anticoagulant (0 1 ml. 38 per cent sodium citrate per 10 ml blood) The blood and anticoagulant are well mixed in the syringe and then run down the side of the silicone tube with gentle pressure on the plunger of the syringe After centrifuging at 5°C for 60 minutes at 2000 g the upper four fifths of the plasma is removed with a silicone-coated dropper pipet and stored at 5°C for testing the same day. Blood from a normal control is collected by the same technique at the same time

3 $CaCl_2$ 2 22 Gm. anhydrous $CaCl_2$ is dissolved in 100 ml. distilled water (0 2 M).

Steps in the Procedure Place 0 1 ml of a 1 per cent purified cephalin suspension in each of two 13 mm. wide silicone coated tubes. To each add 0.5 ml. of the citrated plasma To the first tube add at once 0 05 ml. 0 2 M $CaCl_2$ shake, stopper the tube and measure the clotting time in a water bath at 38°C Shake and stopper the second tube and incubate at 38°C for 30 minutes, then add 0 05 ml. of 0 2 M $CaCl_2$ and measure the clotting time

All glass surfaces (tubes, pipettes) coming in contact with plasma are siliconized

Calculation In order to convert the clotting times to units of antithromboplastin (anticephalin) activity, the response of a pooled stable normal plasma (no less than 5 in the pool) to a standard suspension of cephalin must be worked out This is done by adding to this plasma gradually decreasing amounts of a cephalin suspension, the potency of which in units has been determined (See two-stage method of measuring antithromboplastin activity, page 307) Let us say that the 1 per cent suspension of cephalin is known to have 90 units of cephalin activity per mgm Dilutions of this suspension are tested on the pooled plasma for their clot accelerating activity until they equal the clotting time of the control (0 85 per cent NaCl, instead of cephalin) as follows

suitable for the preparation of cephalin The resulting suspensions have been non-homogeneous and have lost activity during storage. (9) For some cephalin preparations an incubation period of 30 minutes may result in very long clotting times that are not suitable for determining units of anticephalin activity.

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4. Demonstration of Thromboplastin-Inhibiting Activity in Serum and Plasma (Method of Lanchantin and Ware)

Adapted by R. R. HOLBURN*

Principle When barium sulfate adsorbed serum or plasma is incubated with thromboplastin and calcium chloride, a marked inhibition of thromboplastin activity results

Apparatus and Reagents: Saline-imidazole buffer Dissolve 3 Gm Imidazole C P in 975 ml of 0 7 per cent NaCl solution and 25 ml. of 0 5 N HCl pH of this solution is 7.4

Thromboplastin Acetone dried human brain powder is prepared by standard techniques Five gms brain powder are extracted with 100 ml of 0 9 per cent NaCl containing 0 002 M potassium oxalate The extraction is carried out at 45° to 50°C for 30' with occasional stirring Light centrifugation removes the gross particles The crude product is centrifuged for two hours at 28,000 g The pellet is resuspended in oxalated saline to its original volume This is repeated twice more After the last resuspension, the sediment is taken up in saline-imidazole buffer to 10 times its original volume

Prothrombin-free beef plasma (stable source of fibrinogen and factor V). Freshly collected beef blood is added to 0 1 M potassium oxalate (ratio

* From Journal of Clinical Investigation *32* 381, 1953

ing units of cephalin activity are read directly below in the abscissa The units of cephalin activity lost during incubation are obtained by subtracting the units corresponding to the clotting time of the incubated plasma from those of the unincubated. One unit of antithromboplastin (anticephalin) activity is, by definition, that activity of the plasma required to destroy one unit of cephalin activity in silicone coated tubes, after 30 minutes of incubation at 38°C. of the plasma and the cephalin suspension The units of cephalin activity lost are therefore directly converted to units of antithromboplastin activity

Example. Three plasmas, one normal, one hypercoagulable and one hemophilic were tested as described. The clotting times and the calculated results follow:

Type of Plasma	Clotting Time (secs.)		Equivalent Cephalin Units		Antithromboplastin Activity Units
	Unincubated	Incubated	Unincubated	Incubated	
Normal.. .	420	460	107	90	17
Hypercoagulable.	315	325	180	175	5
Hemophilic .	535	1000	68	20	48

Range of Values: Mean of 33 determinations performed on 33 normal men and women: 14.8 antithromboplastin units per ml. plasma ± 4.2 (stand. dev.) The mean value for 20 determinations on 13 Hemophilia A patients was 47 2 units per ml plasma ± 13 8 (stand dev).

Precautions and Sources of Error (1) Defectively collected plasmas are unsuitable for testing, because the evolution of accelerators during partial clotting offsets the action of inhibitors. (2) The plasma must not be frozen or allowed to stand longer than 3 hours at 5°C before being tested (3) The platelet content of the plasma must be less than 4,000 per cu. mm. (4) The ratio of 5 parts of plasma to 1 of cephalin must be maintained in the incubation mixture, since dilution of the plasma will impair its inhibitor potency Most tests for antithromboplastin described in the literature overlook this precaution (5) For best results, the cephalin suspension should be prepared from brain tissue of the same species as that of the plasma being tested (6) Adherence to the order of addition of the reagents is essential (7) If the content of the Ac-G or prothrombin in the plasma is low (below 25 per cent of normal) the results are unreliable. For example if the plasma of a hemophilic A patient contains only 20 per cent normal prothrombin, the incubated mixture may not clot at all, making it impossible to read the values on the cephalin standard curve chart. (8) Some acetone-dried brain powders have been found to be un-

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5. Estimation of Antithromboplastin (Anticephalin) Activity in Plasma (Two-Stage Method)

R. R. HOLBURN and L. M. TOCANTINS

Object To assay the antithromboplastin activity of normal and abnormal plasmas, using cephalin as a thromboplastic agent and the two-stage method of estimating thrombin output as a measure of thromboplastic activity.

Principle Properly collected plasma has the property of reducing the clot accelerating activity of cephalin, after the latter is incubated with the plasma The change in the rate of thrombin output before and after the incubation period will reflect the loss of cephalin activity Using a standard curve of cephalin activity as reference, clotting times are converted into units of antithromboplastin

Apparatus and Reagents (Siliconized glassware used throughout ex- [illegible] fibrinogen is added and uncoated [illegible] Dis- [illegible] Co) [illegible] final [illegible] *Solu-* [illegible] in 100 ml [illegible] 85 per cent NaCl solution Centrifuge [illegible] small amount of the insoluble material. In general, commercial acacia contains about 0 6 per cent calcium by weight (0 023M) It is diluted when added to the incubation substrate to bring its calcium concentration into optimum range (0025M to 01M) (c) *Imidazole buffer* (pH 7 2-7 4) Dissolve 1 72 grams imidazole (Eastman Kodak) in 90 ml of 0 1N HCl and dilute to 100 ml. with water. (d) *Fibrinogen* 50 mg. of fibrinogen (prepared as described on page 227) is dissolved in 5 ml of buffered saline solution (0 85 per cent

9 to 1). After centrifugation the plasma is mixed with freshly precipitated barium sulfate. Equimolar solutions of barium chloride and sodium sulfate are mixed and the precipitate is washed twice with distilled water. Two grams of the centrifuge-packed barium sulfate (dry weight) are added to each 100 ml. of plasma, dispersed and allowed to stand at room temperature for 30′. The barium sulfate is removed by centrifugation and each 100 ml. of plasma run through a Seitz filter of 40-50 per cent asbestos content The plasma is dialysed against 0.9 per cent NaCl until free of oxalate and then is diluted with an equal volume of 0.9 per cent NaCl containing 0.6 per cent imidazole. The pH is adjusted to 7.4 with 0 5 N HCl and the material is frozen

Water bath. 37°C. All reagents are preheated to 37°C.

Steps in Procedure: To 75 x 10 mm. test tubes are added: 0 1 ml prothrombin-free beef plasma, 0.1 ml. reconstituted dried human plasma (1 to 10), 0.1 ml. buffer, 0.1 ml thromboplastin, 0.1 ml. 0.015 M $CaCl_2$ The clotting time is recorded

Unknown samples are substituted for buffer. In incubated studies, the thromboplastin, calcium, and serum are incubated together before the addition of the other two components.

Calculation In the absence of thromboplastin, the system clots in about 500 seconds. When thromboplastin is added the system clots in about 30 seconds Dilution of thromboplastin results in progressively longer times The logarithm of the thromboplastin concentration in this system is a straight line function of the negative logarithm of the clotting time

Incubation of thromboplastin, calcium and. (a) normal human serum results in an acceleration phase in the first few minutes which is then followed by inhibition, (b) barium sulfate adsorbed human serum results in inhibition immediately, and has the capacity of inactivating 95 per cent of the thromboplastin; (c) barium sulfate adsorbed plasma reacts similarly to serum except that accelerators mask the effects somewhat. Sedimentation of inhibited thromboplastin and resuspension in a calcium-free medium resulted in regeneration of most of the thromboplastic activity, indicating the actual inhibition of thromboplastin

Precautions and Sources of Error (1) Accelerator factors are more efficiently removed with barium sulfate when oxalate is present. Therefore oxalate is added to a strength of 0.02 M and freshly precipitated barium sulfate is added to a concentration of 2 Gm per 100 ml of serum. (2) When calcium is omitted from the incubation phase the inhibition of thromboplastin is much less (3) Better success has been achieved in demonstrating inhibition when oxalate-free systems are used

Calculation: The curves expressing the rate of conversion of prothrombin to thrombin of the various samples are plotted on arithmetic ruled paper. The times at which the aliquots were removed from the reacting mixture are plotted along the abscissa, the amount of thrombin formed at each sampling along the ordinate axis.

The amount of thrombin formed is calculated on the basis that 1 unit of thrombin is that amount which clots the fibrinogen solution in 15 seconds under the conditions of the test (table 1). The factor thus obtained is multiplied by the total dilution to give the total thrombin units per ml of

TABLE 1. *Conversion Factors for Calculation of Thrombin Unitages*

Seconds	Factor	Seconds	Factor	Seconds	Factor	Seconds	Factor
11 0	1 50	15 0	1 00	19 0	75	23 0	65
2	1 47	15 2	97	19 2	74	23 2	64
4	1 44	15 4	96	19 4	73	23 4	64
6	1 41	15 6	95	19 6	73	23 6	64
8	1 38	15 8	94	19 8	72	23 8	64
12 0	1 34	16 0	92	20 0	72	24 0	64
2	1 31	16 2	91	20 2	72	24 2	63
4	1 28	16 4	89	20 4	71	24 4	63
6	1 25	16 6	88	20 6	71	24 6	63
8	1 23	16 8	86	20 8	70	24 8	61
13 0	1 20	17 0	85	21 0	70	25 0	60
2	1 17	17 2	84	21 2	69	26 0	49
4	1 16	17 4	83	21 4	68	27 0	47
6	1 13	17 6	82	21 6	68	28 0	44
8	1 12	17 8	81	21 8	68	29 0	43
14 0	1 10	18 0	80	22 0	67	30 0	40
2	1 07	18 2	79	22 2	66	31 0	38
4	1 05	18 4	77	22 4	66	33 0	34
6	1 03	18 6	76	22 6	65	35 0	31
8	1 02	18 8	76	22 8	65	37 0	28

plasma The dilution factor is obtained as follows. $\frac{59}{60}$ (anticoagulant, based on a hematocrit of 40), $\frac{100}{108}$ (defibrination), $\frac{9}{10}$ (cephalin), $\frac{1}{16}$ (saline), $\frac{16}{46}$ (activation substrate), $\frac{300}{375}$ (fibrinogen).

$$\frac{59}{60} \times \frac{100}{108} \times \frac{9}{10} \times \frac{1}{16} \times \frac{16}{46} \times \frac{300}{375} = \frac{1}{70\ 17}$$

The estimation of the thrombin yield per minute is made from the curves for prothrombin conversion (fig 1) The data presented in table 2 are shown in the curves plotted in figure 1 The rate of thrombin output per minute is obtained by dividing the maximum thrombin yield for each plasma by the time in minutes required to reach this value

NaCl containing 5 per cent imidazole by volume). Centrifuge lightly after thawing to remove any insoluble material. (e) *Activation substrate.* 2 parts of 15 per cent acacia in 0.85 per cent NaCl; ½ part imidazole buffer, 3½ parts 0 85 per cent NaCl. (f) *Cephalin.* The purified preparation from human brain powder as for the one-stage antithromboplastin test (see page 300) and used in 1 per cent concentration. (g) *Stable Plasma.* Collected with especial precautions as described on page 302. Siliconized syringes and tubes. (h) *Standard Control Plasma.* Lots of 50 or more small quantities of pooled normal plasma are frozen and used as a standard control for reagent reactivity. After thawing at 37°C. this plasma is treated subsequently in the same manner as the freshly collected plasma.

Steps in Procedure: (1) To 0.1 ml. 1 per cent cephalin add 0 9 ml defibrinated plasma in a siliconized tube. (2) Mix and remove 0 1 ml. of the mixture and add to a siliconized tube containing 1.5 ml. buffered 0.85 per cent NaCl and 3.0 ml. Activation substrate. Start stop watch. Stopper remainder of the plasma-cephalin mixture and allow to incubate 30 minutes at 38°C. (3) At three to four minute intervals remove 0 3 ml of the activated mixture and add to 0 075 ml 1 per cent purified fibrinogen solution. Record clotting time. The endpoint is the appearance of heavy flocculation in the system Aliquots are removed and added to fibrinogen until the clotting time of three successive determinations remains the same. At the end of the thirty-minute incubation period, remove 0.1 ml of the plasma-cephalin mixture and proceed as in steps two and three. It is not necessary to incubate the standard frozen control plasma with cephalin since inclusion of this plasma in the testing is merely a control for reagent activity

If the test plasma should have a prothrombin content of less than 50 per cent, the clotting times of the activated mixture may become inconveniently prolonged It is desirable then to diminish the dilution of the plasma by the activation substrate-buffered 0.85 per cent NaCl mixture from 1 to 46 to 1 to 16. This is accomplished by adding 0.3 ml. of plasma to 1.5 ml. saline and 3 0 ml activation substrate, this will reduce the total dilution from 70 1 to 23 4 and the clotting time from 39 seconds to 19 seconds, for a plasma of 30 per cent prothrombin concentration Changing the dilution factor does not alter the plasma antithromboplastin activity determination If the Ac-globulin content of the plasma should be reduced below 20 per cent, there will be some reduction in the thrombin yield; however, a minor change in the dilution factor will result in a satisfactory decrease in the clotting time, without affecting the rate of thrombin output, since the comparison of the decrease in rate of thrombin formation of the nonincubated and incubated specimens is the basis of measurement of antithromboplastin activity

This result must be corrected by a factor for reagent reactivity, since by definition, the control plasma should produce 5 6 thrombin units/ml. plasma/minute.

Correction factor:

$$\frac{\text{Thrombin units/ml. standard frozen plasma/min. by definition}}{\text{Thrombin units/ml. standard plasma/min in the current test}} = \frac{5\ 6}{5.1} = 1.1$$

TABLE 2: *Time of Sampling and Equivalent Clotting Times and Thrombin Units Developed by the 3 Plasmas*

Type of Plasma	Time of Sampling (Minutes)	Clotting Time (Seconds)	Conversion Factor	Dilution	Thrombin Yield (Units)
Frozen Control	4	37 0	28	× 70 1	20
	6	25 8	.50	× 70 1	35
	8	17 6	82	× 70 1	57
	14	14 8	1 02	× 70 1	71 5
	20	14 8	1 02	× 70 1	71 5
	28	14 8	1 02	× 70 1	71 5
Normal nonincubated	4	29 0	43	× 70 1	30
	7	19 2	74	× 70 1	52
	10	17 8	81	× 70 1	57
	16	16 8	86	× 70 1	60 3
	20	16 8	86	× 70 1	60 3
Normal incubated 30'	5	27 8	45	× 70 1	31 5
	7	25 8	52	× 70 1	36 5
	8	25 4	56	× 70 1	39 3
	10	25 2	59	× 70 1	41 3
	14	25 2	59	× 70 1	41 3
	22	25 2	59	× 70 1	41 3
Hemophilic nonincubated	10	60	—	—	—
	12	34 0	33	× 70 1	23 2
	14	30 5	39	× 70 1	27 5
	16	25 7	51	× 70 1	35 8
	26	25 7	51	× 70 1	35 8
	32	25 8	51	× 70 1	35 8
Hemophilic incubated 30'	10	60	—	—	—
	18	42 0	19	× 70 1	13 4
	22	36 0	29	× 70 1	20 5
	25	33 0	34	× 70 1	24 0
	32	33 2	33	× 70 1	23 1

This factor is used to correct the results obtained in the testing of the fresh plasmas as follows.

Normal plasma. (nonincubated) $\frac{60\ 3 \times 1\ 1}{11} = 6\ 0$

Normal plasma· (incubated) $\frac{41.3 \times 1\ 1}{9} = 5\ 0$

Hemophilic plasma (nonincubated) $\frac{35\ 8 \times 1\ 1}{12} = 3\ 3$

Hemophilic plasma· (incubated) $\frac{23\ 1 \times 1\ 1}{25} = 1\ 0$

$$\frac{\text{Total thrombin formed (units/ml. plasma)}}{\text{Minutes required for complete conversion}}$$

= Thrombin units/ml. plasma/minute

From the curves on figure 1 the thrombin units/ml. plasma/minute are as follows:

$$\text{Control plasma: } \frac{71.5}{14} = 5.1$$

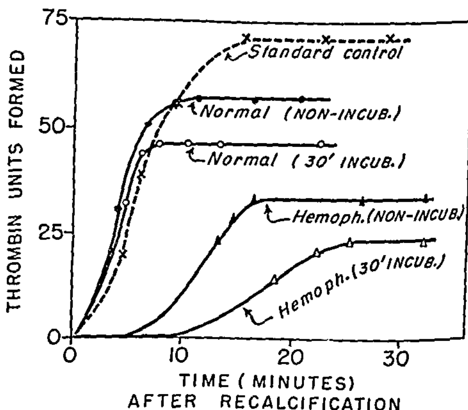


FIG 1.—Anticephalin activity of normal and hemophilic plasmas. The rate of prothrombin conversion in (a) standard control normal plasma, (b) normal plasma non-incubated and (c) incubated with a cephalin suspension before recalcification, (d) Hemophilia "A" plasma non-incubated and (e) incubated with a cephalin suspension before recalcification

From the above curves, the rate of thrombin output per minute is calculated for each plasma. The incubated plasmas form less thrombin and more slowly than the nonincubated ones. The chief object of the standard plasma is to provide a reference control for the reactivity of reagents from day to day

plotted on log-log paper on the ordinate and on the abscissa the mg. of cephalin added per ml. plasma along with the coincident cephalin activity as defined. Cephalin activity is expressed in units, one unit being that amount of cephalin, which under the conditions of the test, will activate standard frozen normal plasma to produce a maximum yield of 0 062 T.U./ml plasma/min The line representing the relation between thrombin output and cephalin activity is shown in figure 2 and is the result of testing of four separately prepared cephalin suspensions. Since the values for each of the four lines were superimposed on one another, the line on figure 2, representing the mean values for the four, was adopted as the standard of reference to express the activity of the cephalin suspensions

Employing the chart in figure 2 as a reference standard the cephalin activities of the plasmas, the curves of which are shown on figure 1, are:

Normal plasma (unincubated): *95* units
Normal plasma (incubated): *77* units
Hemophilic plasma (unincubated): *50* units
Hemophilic plasma (incubated): *6* units

The units of antithromboplastin (anticephalin) activity are the difference between the units of cephalin activity of unincubated and incubated plasmas.

Normal (0′ incub.) — Normal (30′ incub.) = 95 − 77 = 18 units

Hemophilic (0′ incub.) — Hemophilic (30′ incub.) = 50 − 6 = 44 units.

The frozen control plasma functions as an index to reagent reactivity, in addition to its primary purpose of having supplied the substrate for the standardization of the cephalin suspensions. When this plasma is about exhausted a new one is collected, divided into small lots (2 ml) and stored at −20°C The response of the new plasma standard to a cephalin suspension is compared and adjusted to that of the old plasma

Cephalin suspensions, prepared as described, have been uniform in activity when kept at 5°C. for over three months The average rate of thrombin production per ml of plasma (stored frozen) per minute, activated by 1.1 mg. of cephalin is 6 2 thrombin units; this has been regarded to represent 100 units of cephalin activity.

One unit of cephalin activity is, therefore, that amount which will activate a standard plasma to produce 0 062 thrombin units per ml plasma per minute. One unit of antithromboplastin (anticephalin) activity is that which, after incubation of the plasma at 38°C for 30 minutes as described, will reduce by 0 062 units the capacity of the cephalin suspension to activate the conversion of prothrombin in a plasma Or, in other terms, one unit of

The next step is to convert the thrombin units per ml. plasma per minute into units of cephalin activity. This is done by referring to the standard chart (fig. 2) which expresses this activity in terms of thrombin units per ml. plasma per minute.

Derivation of the Standard Curve of Cephalin Activity: A 1 per cent purified human cephalin suspension is diluted with 0.85 per cent NaCl solution from 1.0 to 0.1 per cent concentration. A 0.1 ml. aliquot of each dilution is added to 0.9 ml. of the defibrinated standard control plasma (kept frozen) and the two-stage test procedure followed. The values for the rate of thrombin formation (thrombin units/ml. plasma/min.) are

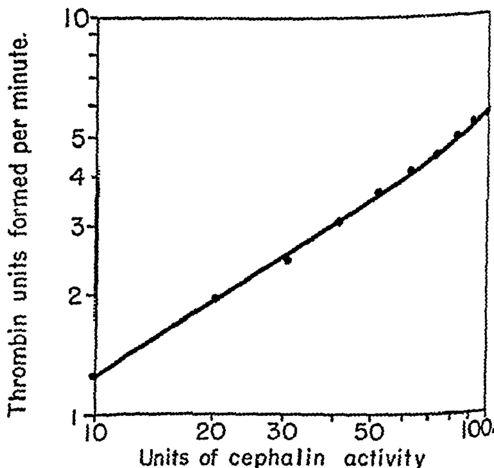


FIG 2.—Standard curve for two-stage estimation of cephalin activity

6. Assay of Prothromboplastic and Antithromboplastic Activities

J. H. FERGUSON

There are a number of ways to convert prothrombin to thrombin experimentally. In most, if not all of them, a key role in the activator mechanism depends upon *phospholipid*, in one form or another. *Extrinsic* activation is a convenient term for systems employing "tissue thromboplastin(s)," or factor III, thus presumably corresponding to the natural clotting of blood when it comes into contact with injured tissues Interactions between factors in tissue thromboplastin and in plasma result in thrombin formation. Biochemically, tissue thromboplastins are crude, multicomponent, particulate (? macromolecular), lipoprotein complexes, as yet insufficiently resolved for us to state exactly what it is that determines their "thromboplastic" activity. They do lose this activity when deprived of their lipid fraction. Several enzymatic activities have been observed, on occasion, with various factor III preparations *Intrinsic* activation, on the other hand, refers to an activity associated with the blood itself. A significant contribution normally comes from the platelets Platelets are rich in lipids, including phospholipids. It is possible, with some difficulty, to prepare mammalian plasma so free from all formed elements (including platelets and their fragments) as to be incoagulable on simple recalcification Clotting is restored, however, on adding either platelets or cephalin [1] The intrinsic mechanism, therefore, involves interactions between platelets (or phospholipids) and plasma factors The so-called (TGT) thromboplastin generation test (p. 89) is one way to study these interactions The new two-stage (thrombin generation) method(s),[2] to be described in this chapter, offer another approach to these problems.

Definitions and Methodology The term "thromboplastin" has been used widely, with various implied meanings, none of which are sufficiently definitive We suggest that it is better to use the adjectival "thromboplastic," for *processes* (rather than substances) having the common features of (a) specific activity in connection with the conversion of prothrombin to thrombin, and (b) dependence of this upon phospholipids, in some form or other We propose the term "prothromboplastic phosphatide" for any phospholipid (capable, eventually, of biochemical identification) determining the above "thromboplastic" activity Here, we will not deal in any detail with tissue (extrinsic) thromboplastic systems, but will limit our consideration to test systems which use isolated ("free" or "available") phospholipids These, particularly in their cruder forms (e g, platelets, or

antithromboplastin is that amount which will inactivate one unit of cephalin activity.

The fundamental criterion in the evaluation of inhibitory activity of plasma is the stability of reagents. Cephalin, if prepared as described in this technique, has the property of maintaining its clot accelerating activity when incubated alone for 30 minutes at 37°C. while some thromboplastin preparations do not. Cephalin has additional advantages in that it can be purified, weighed to insure exact concentration and stored at 5°C. without loss of activity for several months.

A properly collected plasma, kept away from glass surfaces is essential for reliable results, since unstable plasma will lead to rapid conversion of prothrombin to thrombin.

It is necessary to keep the ratio of cephalin to plasma 1 to 9 in order to have high inhibitor concentration in the plasma-cephalin mixture during incubation.

Values Obtained: In 39 determinations on 39 normal adults, the mean antithromboplastin activity by this two-stage method was 12.8 ± 4.3 (stand. dev.) units. In 24 tests on 13 hemophilic patients, the mean value was 51.0 ± 16 4 (s.d.) units

Precautions and Sources of Error. (1) Contact of plasma with glass or adsorbants, i e., asbestos, clay, results in a hypercoagulable type of plasma, in which, after sufficiently long period of contact, it may not be possible to distinguish between the antithromboplastin activities of hypercoagulable, normal and hemophilic plasmas This is true also of plasmas resulting from poor venepunctures (2) Prothrombin and ac-globulin content should be kept within a practical range by dilution during the measurement of thrombin activity in order to avoid unduly prolonged clotting time

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[5] ——— Estimation of the anticephalin activity of whole blood Proc Soc Exper Biol & Med. *57*, 211-215, 1944

6. *Assay of Prothromboplastic and Ant thromboplastic Activities*

J. H. FERGUSON

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the newer clotting factors.[2] The basic system, moreover, lends itself to the testing of many experimental additives, the study of which can shed considerable light upon the fundamental mechanisms of blood clotting.[3] By preincubating groups of factors,[3] various intermediate and side reactions can be investigated. Not only "activator" properties, but also a variety of "inhibitor" effects may be explored with these methods. In the following, we show particular applications to the qualitative and quantitative study of *prothromboplastic* and *antithromboplastic* agents.[4] In this use, several modifications of the test system (Stypven-, trypsin-, intrinsic-)[5] may be compared, and essential similarities of the results may be considered confirmatory of fundamental principles which they all exemplify. Properly conducted, these tests are reliable, specific, and extremely sensitive.

Stypven-activated eluate two-stage. This modification will be presented first, and in detail, since it seems to be the easiest, most reproducible, and most sensitive.

Materials: Eluate (prothrombin, etc.) Platelet-poor *plasma* (human, bovine, or canine) is obtained by following, as closely as possible, the principles given in Chapter 1. For example, a normal human donor typically provides a "half unit" of blood, thus, using the silicone technic, 225 ml of arm-vein blood are received into 25 ml 0.1 M sod. *oxalate* (not citrate) in a chilled siliconized Erlenmeyer flask, mixed, and transferred to six chilled siliconized lusteroid tubes (50 ml. capacity), balanced, and immediately centrifuged at 4°C (refrigerated centrifuge) for 30 minutes at 3000 rpm. The upper two-thirds of the plasma layer is carefully removed with a bulb-operated, siliconized 5 or 10 ml pipette, pooling in a siliconized measuring cylinder (100 ml.) until 90 ml. is secured. Thirty ml. amounts are measured onto 3 Gm. of $BaSO_4$ in each of three lusteroid tubes, stirred with a glass rod for 10 minutes, then centrifuged at 3000 rpm for 10 minutes. The sediment, after decanting the supernatant, is washed 3 times by filling the tube with *distilled water*, stirring thoroughly, and recentrifuging. Then 10 ml. of 0.2 M sod. citrate is added to each tube, stirring thoroughly to elute, then centrifuging to recover the *eluate*. The pooled eluates are finally dialyzed, in Visking casing, at room temperature, overnight (rocking dialyzer), against 0.85 per cent NaCl containing 1/200 volume of 1 M sod. citrate. If there is any sediment this is removed by centrifugation. The final preparation is then distributed in 2-5 ml. lots, in small lusteroid tubes, and stored frozen in the deep freeze at −20°C. Bovine or canine preparations are equally satisfactory and have been used in much of our investigative work. Just before use, a tube of the stock eluate is thawed, filtered or centrifuged (if there is any sediment), and tested. Amounts of eluate not used in the day's tests can fre-

"fraction 3"), erythrocyte stroma ("hemolysate"), cephalin, soybean phosphatides, etc.), have also been called "*partial* thromboplastins," compared with "*complete* (tissue) thromboplastins," recognizing, of course, that *both need plasmatic (accessory) factors*.

The two-stage (thrombic) system, by which we propose to study "thromboplastic" activities, will now be described in principle and in practice. Any thrombic two-stage system consists of a first step, in which prothrombin is incubated with the necessary activators, and a second step, in which the thrombin formation is followed by noting the clotting-times when successive incubate samples are added to a test fibrinogen. Other things being equal, *shorter clotting times mean more thrombin*. The oldest such method, which is still used in our procedure, employs a constant (initial) amount of prothrombin, in a fixed total volume of incubate, and follows the course of thrombin formation to its completion in each incubate. A variety of prothrombin preparations may be used, as long as all necessary activators are provided Our practical routine now uses simple plasma *eluates* (see *Materials*), supplemented by suitable sources of needed accessory factors (see *Materials*) As thrombin formation proceeds in the incubate, the second stage clotting-times, when plotted graphically against the incubation periods, form an "activation curve." The most significant data are three, namely, (1) the first (usually 1 minute) test clotting-time, which indicates the initial reaction rate, (2) the optimal incubation period, which indicates the over-all reaction rate and is measured as the time (minutes) required to reach the optimum, (3) the optimum (end point, or shortest clotting time reached), which measures the maximal thrombin yield possible under the particular experimental conditions

When only the *prothrombin* is varied, and particularly when tissue thromboplastin is used (our "extrinsic" modification), the method offers a very reliable means of prothrombin assay [1,2] It is also possible to use the basic method adapted, in turn, to the quantitative study of any other clotting factor. All that is necessary is to test the reagents for all known clotting factors and ensure that they are *all under simultaneous quantitative control* Thus, we secure a deficiency of just one factor at a time and then test the effects *specifically* attributable to restoring measured amounts of the missing factor In practice, the deficiency need not be absolute, but must be enough to give defective thrombin formation in the control and a significant improvement with each addition of the factor in question Technical details must be worked out in each case and more investigation will be needed before sufficiently standardized procedures become available for every known clotting factor The principle, however, has been established, and feasibility in practice has been demonstrated for a number of

Calcium (Ca) is 0.15 M $CaCl_2$.

Fibrinogen Any well-standardized prothrombin-free fibrinogen s tion is satisfactory. We routinely use Armour's bovine fibrinogen (f tion I), lyophilized, of which we have a large stock in use for a numbe years Periodically, a solution is prepared as follows: 100 ml. *b. sal* used per 1.5 Gm. fibrinogen, gently stirred over several hours, then filt to remove insoluble material. Each 100 ml is adsorbed with 1 Gm Ba for 10 minutes, centrifuged, and the supernate distributed in 5-10 ml for frozen storage at −20°C. Thawed in the water bath (37°C.) be use, the solution seldom needs filtering, but any sediment should be removed The frozen stocks remain serviceable for many months and particular material seems to have a well-stabilized "reactivity," as sh by the repeated securing of test clotting times of very similar values 1:2 dilution with *b. sal.* just before use is preferred for the clotting te which should be easy to read accurately as regards the fibrin forma "end point"

Other agents, used to exemplify applications and interpretations of method, will be mentioned briefly under *Results*

Method. For the stypven modification of the two-stage method, *incubate* consists of 4 0 ml *b sal*, 0 1 ml eluate, 0 1 ml 1 10 A 0.1 ml cephalin dilution (*cf* saline control, and "unknown"), 0 2 1.10,000 stypven ("optimal"), and finally 0 5 ml 0 15 M $CaCl_2$ at *start* of incubation

Clotting-tests, the second stage, are made by removing 0 2 ml samp after successive minutes of incubation, adding to 0 2 ml fibrinogen, timing the first visible fibrin threads (not mere turbidity) with a stop wa

Incubation and testing are routinely conducted at a reasonably st room temperature (26° ± 2°C) All glassware should be scrupulo clean (p 8) Blow-out volumetric pipettes and 13 × 100 mm (Was mann) tubes are used for the clotting tests

Results and Interpretation Of the three data parameters (p 316), *end point* (optimum) is determined with the greatest possible accuracy its use in quantitative bioassays of phospholipid reactivities in the styp eluate system is as follows

On plotting clotting-time end points (seconds) against phosphol concentration (*μg per ml incubate*) *on log-log* paper, *essentially rectili* plots are obtained over the concentration range suitable for bioas Figure 1 shows *parallel* straight line plots for the standard cephalin (C B) and an "unknown" lipid (PS-3) which, in this case, was a highly p fied phosphatidylserine prepared from human platelets by Marcus et *Calculation* For each test, the observed end point clotting time of amo (N) of the unknown is read off the appropriate standard as the amo

quently be refrozen and used on a subsequent occasion Many of our stock eluates have remained serviceable *and given reproducible results (see below)* for several months

If only *citrated* plasma is available, for instance in the case of special substrates from clinical cases, it is possible to use $Al(OH)_3$-phosphate (dialzyed) eluates instead. As these are not routine, details will be omitted.

Normal eluates provide prothrombin (factor II), accompanied by sufficient factors VII, IX, X, and, probably (although these remain to be quantitated) XI (PTA) and XII (Hageman), but, at most, only traces of factors V and VIII. For the *stypven* two-stage (besides prothrombin, prothromboplastic phosphatide, and calcium), only factors V and X are needed Factor X is in the eluate, and V is provided in the following "AcG" preparation.

"AcG" ($BaCO_3$-adsorbed beef serum, according to Ware and Seegers), is prepared as follows: Beef blood, collected in bulk at the slaughter house, is kept overnight in the refrigerator (4°C.) and then centrifuged for serum This "aged" serum is adsorbed for 10 minutes with 40 mg./ml $BaCO_3$, recovering the supernate by centrifugation and distributing it in 2-5 ml. lots for frozen storage at −20°C. Before use, a unit of stock is thawed and diluted 1.10 with imidazole-buffered saline (*b. sal.*: which is made by adding 1 volume 1 72 per cent imidazole-HCl, pH 7.2-7.4, to 9 volumes 0.85 per cent NaCl). Although the stock "AcG" remains potent for months, or even years, in the frozen state, it seems a wise routine to avoid possible change in V-activity of the 1 10 reagent by keeping the tube in a melting-ice bath during the experiment, and to prepare a new dilution on each occasion. The reagent is admittedly crude (especially containing considerable amounts of "activated" Hageman-PTA), but only its factor V content is of significance in the Stypven test used for the present purposes.

Stypven is the commercial (Burroughs Wellcome) preparation of Russell's viper venom, which is used in a 1 10,000 solution in distilled water This solution keeps well in the refrigerator (4°C) for many days.

Cephalin (Ceph) is our "standard" prothromboplastic agent It is the relatively crude $CHCl_3$-extract of acetone-dried *human* brain, according to the method of Bell and Alton (1954) One Gm of dried brain powder is extracted for an hour with 50 ml $CHCl_3$, then suction-filtered through a Buchner funnel, evaporated, weighed, and suspended (homogenized*) in 0.85 per cent NaCl to a 1 per cent stock solution One to 2 ml aliquots are frozen stored at −20°C and retain full activity for many months For use, the necessary amount is thawed and diluted (1 10, etc.) with *b. sal.* (above).

* *Questions of* homogenization and the physical state, stability, etc. of phospholipids need *further* investigation, but will not be pursued in tl

Calcium (Ca) is 0 15 M $CaCl_2$.

Fibrinogen. Any well-standardized prothrombin-free fibrinogen solution is satisfactory We routinely use Armour's bovine fibrinogen (fraction I), lyophilized, of which we have a large stock in use for a number of years. Periodically, a solution is prepared as follows: 100 ml. *b. sal.* is used per 1.5 Gm. fibrinogen, gently stirred over several hours, then filtered to remove insoluble material. Each 100 ml. is adsorbed with 1 Gm. $BaSO_4$ for 10 minutes, centrifuged, and the supernate distributed in 5-10 ml. lots for frozen storage at −20°C. Thawed in the water bath (37°C.) before use, the solution seldom needs filtering, but any sediment should be so removed. The frozen stocks remain serviceable for many months and our particular material seems to have a well-stabilized "reactivity," as shown by the repeated securing of test clotting times of very similar values A 1:2 dilution with *b. sal.* just before use is preferred for the clotting tests, which should be easy to read accurately as regards the fibrin formation "end point"

Other agents, used to exemplify applications and interpretations of the method, will be mentioned briefly under *Results.*

Method For the stypven modification of the two-stage method, the *incubate* consists of 4 0 ml *b sal*, 0 1 ml eluate, 0.1 ml 1:10 AcG, 0.1 ml. cephalin dilution (*cf* saline control, and "unknown"), 0.2 ml. 1 10,000 stypven ("optimal"), and finally 0.5 ml 0.15 M $CaCl_2$ at the *start* of incubation.

Clotting-tests, the second stage, are made by removing 0 2 ml samples, after successive minutes of incubation, adding to 0 2 ml fibrinogen, and timing the first visible fibrin threads (not mere turbidity) with a stop watch.

Incubation and testing are routinely conducted at a reasonably stable room temperature (26° ± 2°C) All glassware should be scrupulously clean (p 8) Blow-out volumetric pipettes and 13 × 100 mm (Wassermann) tubes are used for the clotting tests

Results and Interpretation Of the three data parameters (p 316), the *end point* (optimum) is determined with the greatest possible accuracy and its use in quantitative bioassays of phospholipid reactivities in the stypven-eluate system is as follows

On plotting clotting-time end points (seconds) against phospholipid concentration (μg per ml incubate) on *log-log* paper, essentially rectilinear *plots are obtained over the concentration range suitable for bioassay* Figure 1 shows *parallel* straight line plots for the standard cephalin (Ceph. *B*) and an "unknown" lipid (PS 3) which, in this case, was a highly purified phosphatidylserine prepared from human platelets by Marcus et al.[5] *Calculation* For each test, the observed end point clotting time of amount (N) of the unknown is read off the appropriate standard as the amount

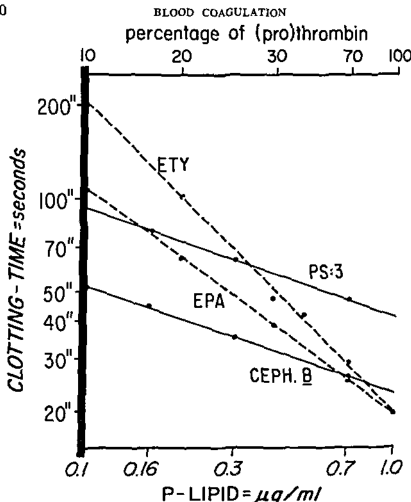


FIG 1.—Assay of phosphatidylserine (PS 3) in terms of cephalin (Ceph *B*), EPA, or ETY.

(μg /ml) of standard (cephalin) with equivalent reactivity—that is, giving the same end point. The assay value, as percentage of the standard is: (Ceph. Eq. − N) X 100 In the cited example, the PS assayed close to 20 per cent.

The EPA and ETY lines of figure 1 will be discussed later.

Suitability of cephalin standards. Exhaustive studies[4] gave the following results:

1. Replicate tests on the same occasion, and similar tests on different occasions (over a 3-month period) established a fixed reproducibility of the Ceph results, within a small (± 5-10 per cent experimental error, and with few exceptions (2-4.). Valid data were obtained with Ceph: concentrations between 1.0 and 0 1 μg /ml

2. Occasionally, the plots, although parallel to the *fixed* standard, *averaged* from 1., were somewhat beyond the experimental error, suggesting some change in test conditions. It is common to many bioassays to obtain a "standard-*of-the-day*" and to make a small correction for any change in conditions. The addition of a little sodium desoxycholate, needed to homogenize certain phospholipid preparations, is an example of change in conditions.

3. Divergent (non-parallel) plots invalidate the bioassay. These were obtained under two conditions: (a) when the phospholipid is incompletely homogenized, and (b) when it is weak and exposed to air for long periods (presumably causing "oxidative" alterations). The remarkable finding under (b) was that this caused an anomalous *hyper*reactivity in the weaker concentrations. Thus, the data plot remained rectilinear (unless alterations continued during the test), but with a different slope (less steep).

4 A series of problems concerning changing reactivities of individual phosphatides is opened up by these studies and the new method offers a tool for further investigations Some simpler deteriorative losses in potency are assayable

Reactivities of mixtures of Phospholipids in the Stypven-eluate test system Cephalin is a relatively crude mixture of phosphatides The finding that it was considerably more active, weight-for-weight, in validated assay comparisons with the best available purified platelet phospholipids, invited exploration of the results obtainable with mixtures of phosphatides.

Method It is implicit that the end points of the two-stage technic measure the amounts of prothrombin converted to thrombin With suboptimal amounts of phosphatides, we are dealing with incomplete activations, which, in all probability, represent equilibrium reactions Additional frames of reference are available, if we can quantitate these equilibria This requires correlation of the end point clotting-times with the amount (percentage) of prothrombin activated, or of the potential (optimal = 100 per cent) thrombin yield Figure 1 illustrates how this was accomplished

Equivalent prothrombin activations (EPA) Serial dilutions of eluate (with factor V, previously mixed), corresponding to 100, 70, 40, 20, and 10 per cent of the original (100 per cent strength, were maximally activated with optimal (20 μg /ml) Ceph in the usual Stypven test system. Figure 1, line EPA, shows that the log-log plot of these end point clotting times against the above precentages of "prothrombin" was precisely rectilinear These data were confirmed on other occasions

Equivalent thrombin yields (ETY). In other experiments, full-strength (100 per cent) eluate was maximally activated to "thrombin," using 20 μg /ml Ceph in the Stypven test system Then various dilutions (percentagewise) were immediately retested on the same standard fibrinogen.

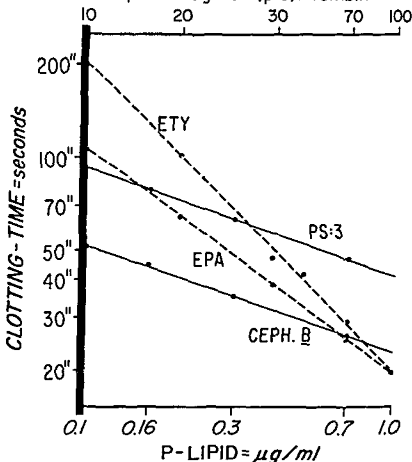


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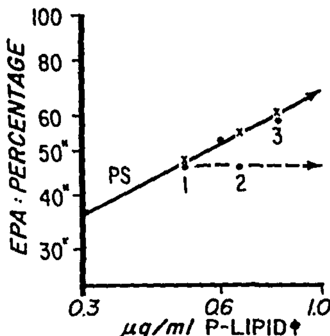


FIG 2—Tests for antithromboplastic activity in three mixtures (1, 2, 3) of a lipid inhibitor (LI) with phosphatidylserine (PS) See text EPA percentages plotted against "expected" (x) and "found" (•) equivalents of phospholipid

solid line); (b) expected summations (indicated x) of (1) 0 3 PS + 0 3 LI, (2) 0 3 PS + 0 6 LI, and (3) 0 6 PS + 0 3 LI (µg./ml); (c) found values (indicated, •) for the actual mixtures.

The last were all less than expected, and the effects of increasing the LI from (1) to (2) are emphasized by connecting these two points with the interrupted line in figure 2 The method distinctly reveals inhibitory effects of the antithromboplastic lipid in as little as 0.3-0 6 µg /ml, which is a high degree of sensitivity

The absence of any indication of inhibitory effects with the platelet PS or PE preparations may be regarded as physiologic evidence for the high purity of these phospholipid products It remains to be proved whether the inhibitor demonstrated in the brain "phosphatidylserine,"[6] or LI, may be due to some contaminant

Reactivities of phospholipids in other clotting test systems. The foregoing illustrates some of the variations in reactivity which depend on the particular phospholipid The other variable is the test system, since it may very well be expected that different systems, involving certain diversities of

Figure 1, line ETY, gives our best experimental data. These showed variability in repeated tests, however, which was attributed to the well-known experimental difficulty of verifying the so-called inverse law in actual practice. This is discussed on page 18 in ref.[1] The ETY plot of figure 1 obeys the inverse law, but its divergence from the EPA plot is unexplained Notwithstanding these reservations, the ETY results will be cited as essentially confirming the main conclusions drawn from the more reliable EPA data.

Results on mixtures. Table 1 assays two mixtures of PS:3 and Ceph *B*, the mutually valid assay equivalents of which are available from figure 1. The data include: (a) concentrations in μg./ml. (b) clotting-time end points (seconds), (c) cephalin *B* equivalents (Ceph. Eq.), (d) phosphatidylserine equivalents (PS Eq), (e) EPA, and (f) ETY (*see above*). The "found" values are read directly from the reference plots of figure 1. The "expected" values are obtained by adding the common (*same* lipid) equivalents and then reading the values on the reference plots.

TABLE 1· *Analyses of Mixtures of Cephalin (Ceph.) and Phosphatidylserine (PS)*

Test	P-Lipid	C T.	Ceph Eq	PS Eq	EPA	ETY
3-12-62	(μg /ml)	(sec)	(μg /ml)	(μg /ml)	%	%
A.	Cep (0 3)	35 2	0 3	(1 4)	45	54
B.	Ceph (0 16)	44 0	0 16	(0 77)	33	44 5
C	PS (0 7)	45 8	(0 145)	0 7	31 5	43
D.	Ceph (0 3)					
	PS (0 7)	31 1	(0 42)	(2 0)	53	62
E, a.	expected	(30 7)	0 43		54	63
b	"	(30 5)		2 1	54 5	63 5
F.	Ceph (0 16)					
	PS (0 7)	34 5	(0 315)	(1 5)	46	56
G, a	expected	(35 0)	0 305		45	55 5
b	"	(35 0)		1 47	45	55 5

The results show very close agreements of the found and expected values, both for the two equivalents and for the EPA and ETY values. This would not be true if there were any potentiation or antagonism (inhibition), or if the assays were invalid These analyses, therefore, permit the important conclusion that the present assayable (and dilute) phospholipids simply *summate* their prothromboplastic reactivities in a strictly quantitative manner

Assay of antithromboplastic activity This is illustrated in figure 2 for mixtures of the above phosphatidylserine with a pork-brain lipid inhibitor (LI) prepared in the Jefferson Medical School laboratories[6] The tests were conducted in the presence of sodium desoxycholate Figure 2 shows the EPA percentages for (a) increasing concentrations of PS alone (the

7. Preparation and Assay of Blood Antithromboplastin

R. T. CARROLL and L. M. TOCANTINS

Reagents and Apparatus—These are the same as described for the extraction and assay of tissue antithromboplastin (page 376).

Extraction· The blood is withdrawn swiftly (rate of withdrawal no less than 0.5 ml. blood per second) from a well distended vein through a #18 gauge needle, into a siliconized syringe. When the correct volume has been removed, the syringe is detached from the needle and the blood added directly to a large uncoated Erlenmeyer flask containing absolute methanol, the amount having been previously adjusted to correspond to forty times the volume of the blood sample removed. A quick, accurate venepuncture is essential, thereby eliminating, as much as possible, the possibility of contamination of the blood with tissue juices The blood should be squirted directly into the methanol, and not allowed to touch the glass walls of the vessel, before mixing with the reagent.

As the blood is added, the flask is continuously swirled gently, to avoid "caking" of the blood in the alcohol and the resulting decrease in surface area exposed for the extraction The final mixture should contain fine, granular, small reddish particles, which disperse evenly throughout the solution on gentle swirling. The mixture is now allowed to remain at 5°C for a period of 5–7 days Once daily, during this period, the flask is removed from the refrigerator and its contents mixed by swirling in a rapid rotating motion, for a period of approximately two minutes At the end of the extraction period, the mixtures are filtered through two thicknesses of Whatman #2 filter paper The residue is rinsed with two 125 ml portions of absolute methanol The filtrate is then placed in a distilling flask immersed in a water bath maintained at 45–48°C The residue remaining in the distilling flask, upon removal of all the methanol consists of an aqueous and a solid phase To the distilling flask is added absolute ethyl ether, in 20–25 ml volumes, the flask is shaken for three minutes and the mixture placed in a separatory funnel Addition of ether to the flask is repeated until no residue is left in the distilling flask This usually requires between 80 and 100 ml of absolute ether The ether-residue mixture is allowed to remain at room temperature for twenty minutes This permits the separation of two phases, a clear yellow green upper layer and a dark green, almost black lower layer The lower layer is removed with approximately the lower tenth of the upper layer. This is then shaken in a separatory funnel with 30 ml absolute ether Again, after a 20-minute wait, the

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lower layer is removed in a similar fashion and the upper layer is combined with the first upper layer sample This process is repeated once more and the combined upper layers placed in a weighted beaker and the ether solution removed in vacuo. Allowing the ethyl ether-methanol residue mixtures to stand in the separatory funnel at 5°C. for longer periods results in no appreciable difference in the quantity or quality of the lipid inhibitor. This is in contrast to the brain methanol extracts which contain material somewhat soluble in ether at 15-20° but not soluble at 5°C., which exert a coagulant action when tested on plasma.

Testing of Extract. If it is desired to test the material directly, the beaker is first weighed accurately and the weight of the residue obtained by subtracting the initial weight of the beaker. To the product is added sufficient 0.85% NaCl to prepare a 1 or 2 per cent solution. In these concentrations, the unknown blood residues are conveniently prepared and homogenize well. Usually the gravimetric yields are so small (unless large volumes of blood are initially employed) that the final residue obtained, when mixed with the proper amount of 0 85 per cent NaCl, does not lend itself to homogenization with the supersonic vibrator which requires a minimum volume of 5 ml The residue is mixed well with the saline solution, using a rod. The blood residues are much more easily dispersed in 0.85 per cent NaCl than the tissue residues The pH of the lipid suspensions is then adjusted and they are passed through the hand operated homogenizer 5 times and exposed to the supersonic vibrator for a period of 20 minutes. The blood extracts, when suspended in saline solution and homogenized, retain their state of fine dispersion for a longer period than does the tissue inhibitor, possibly indicating the presence of a suspension stabilizing substance in the blood extracts not usually found in the tissues. If the unknown residue is required to stand over a period of 6–8 hours before being tested, it is rehomogenized by exposure to the supersonic vibrator for a period of 20 minutes.

The testing mixture is essentially the same as that described in testing the tissue inhibitor, i e , 0 1 ml unknown fraction, 0 1 ml thromboplastin, 0.1 ml normal citrated plasma, 0 1 ml 0 02 M $CaCl_2$ added in the order named.

The thromboplastin solution used is the same as that described in the assay of the tissue inhibitor (page 378), taking care to use a thromboplastin from the same species as that of the inhibitor and the plasma used as a substrate Since the antithromboplastin obtained from blood is less potent than that obtained from tissues, a better assay of the inhibitor results if a thromboplastin less potent than that used for assay of the tissue inhibitor is used Dilution of the brain thromboplastin 1 to 20 and possibly 1 to 30

with 0 85 per cent NaCl makes it possible to obtain a better quantitative differentiation of the various fractions tested

The plasma used in the testing mixture is obtained in the manner described for that used in testing the tissue inhibitor, care being taken not to use a plasma sample that is older than 12 hours. Close attention must also be paid in this assay to the amount of $CaCl_2$ added to the citrated plasma, to reach the optimal clotting rate. It is important that this is found for each lot of plasma being used as a substrate, since both over or under recalcification may vitiate the results. If native blood or plasma is being extracted, there will of course not be any citrate in the extracts. If citrated or oxalated blood is the source, provision must be made in the testing to offset the effect of any citrate or oxalate carried through the extraction The technic of determining the calcium concentration required for optimal clotting is as follows· Using as reagents those already described, a mixture consisting of

0 1 ml suspension of extract
0 1 ml thromboplastin
0 1 ml normal citrated plasma
0 1 ml $CaCl_2$ (of varying molarity)

is prepared. The molar concentrations of $CaCl_2$ employed are usually 0 01, 0 0125, 0 015, 0 0175, 0 02, 0 0225, 0 025, 0 0275, 0 03, 0.0325, 0 035 The concentration which yields the shortest clotting time is considered as optimum for that clotting mixture.

Assay of the blood extract is carried out exactly as described for the tissue lipid antithromboplastin (page 377) By comparing the clotting time of the unknown with that of the standard, the number of units of antithromboplastin activity per mgm of the extract may be found To find the total unitage, the units per mgm are multiplied by the yield in mgms The total units divided by the number of ml. of blood used, will give the units per ml of blood

Example 20 ml of blood were extracted with 800 ml of absolute methanol The final residue weighed 37 mg A 1 per cent suspension was prepared and gave a clotting time in the activated clotting mixture of 66 seconds One mg of the extract being assayed contained, therefore, 1.96 units in terms of the activity of the reference standard The total activity of the extract was $1\ 96 \times 37 = 72\ 5$ units

$\frac{72\ 5}{20} = 3\ 62$ units of antithromboplastin activity per ml of blood

With every extraction of an unknown blood sample, an extraction of normal blood is performed Every step including the testing is carried out

simultaneously on both samples. The activity of the unknown may then be expressed in terms of per cent of normal. *Example:*

Units of antithromboplastin activity per ml. normal blood: 1.4

Units of antithromboplastin activity per ml. unknown blood· 1.96

The unknown has 140 per cent of the activity of the normal

Sources of Error: (a) Poor venepuncture; admixture of tissue juices (b) Failure to add rapidly sample to methanol and to swirl flask as blood is added, thereby getting "caked" blood. (c) Failure to remove entirely the ether soluble layer or to separate adequately the aqueous from the ether soluble phase. (d) Failure to have an adequately large volume for the supersonic vibrator. (Minimal amount that should be used = 5 ml.)

Preparation and Assay of Plasma Antithromboplastin· Collection of sample. The sample of blood is obtained by means of the technic described in detail elsewhere Siliconized glassware is used throughout and the blood is centrifuged in a refrigerated centrifuge at 120,000 total g (g per minute × no. of mins.). The upper ⅘ of the plasma layer is carefully separated and stored in silicone coated stoppered tube.

Preparation of plasma sample. Visking casing (1″ in diameter) is cut into approximately 14 inch lengths. The lower four inches is moistened under tap water until it becomes sufficiently pliable, and then three tight knots are tied in this using the casing material for the tie To the open end of the sac is added a measured amount of plasma to be extracted The portion of the sac projecting above the plasma meniscus is folded over and tied The sac is then suspended vertically from a crossbar or hammock fashion, between two stands A revolving fan is placed facing the suspended plasma. In this manner a 10 ml sample of normal plasma may be dried in five to six hours.

Extraction of the Plasma The sac containing the dried plasma is removed from the crossbar and the material distal to the two knots is cut off and discarded The remainder, using sharp scissors, is then cut into small pieces (approximately 3 × 5 mm) These are transferred to a mortar and to them is added 50 to 75 ml. absolute methyl alcohol The mixture is then macerated with a pestle by means of both a rotary and a tamping motion, for 7 to 10 minutes The fluid is removed to an Erlenmeyer flask by decantation and a fresh batch of absolute methanol added. The above process is repeated until a volume of methanol corresponding to approximately 30 times the initial plasma volume has been added. Following this approximately 10 additional volumes are employed in transferring the solid material to the Erlenmeyer flask, rinsing the mortar and pestle, etc Finally a total of forty volumes of absolute methanol will have been added for each volume of liquid plasma. The residue now contains small discreet, well dispersed particles which lend themselves well to extraction. This is

allowed to remain at 5°C. for five days. The details of filtration and distillation of the extract and processing, obtaining and homogenizing the residue are as described for the tissue lipid antithromboplastin. In assaying the activity of the extract, allowance must be made for any citrate present, by finding the $CaCl_2$ concentration which gives the optimal clotting time in the activated clotting mixture. A plasma sample obtained from a normal subject is extracted, processed and assayed side by side with the unknown sample.

Results are calculated as described in Chapter XII, Section 4, and expressed either in units of antithromboplastin activity per ml of plasma or in terms of per cent of the normal.

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8. *Measurement of Plasma and Serum Antithrombin Activity*

J. F. JOHNSON and W. H. SEEGERS

There are at least four known natural mechanisms for the inactivation or modification of thrombin action There may be more, but these have been demonstrated or can be measured They are. (1) The adsorption of thrombin on fibrin Although the amount adsorbed in whole blood or plasma is probably not great, this effect is marked in purified systems (2) Heparin co-factor, present in plasma, interfering with the action of thrombin and fibrinogen and thus acting as an antithrombin, though probably not a destroyer of thrombin in the strict sense (3) A substance in plasma and serum that inactivates thrombin serving to remove it from

the clotting system (4) A property of both plasma and serum that is active in the removal of thrombin and requires the activation of prothrombin to manifest itself.

In considering these effects it has been found convenient to refer to them respectively as antithrombin I, antithrombin II, antithrombin III and antithrombin IV. The third action is also known as the natural antithrombin and is the one with which we are concerned in this explanation of the measurement of its strength.

Materials and Reagents: (a) *Thrombin.* To reduce to a minimum extraneous protein interactions which might lead to faulty results, only the purest thrombin available is used as a substrate. This is prepared from purified prothrombin in the manner described in another section (page 181). It is possible to use commercial thrombin that has been carefully and repeatedly checked for its activity Thrombin may possibly change its activity when stored at refrigerator temperatures, even becoming more active overnight. A constant check is necessary, particularly when commercial products are used in these analytical procedures.

Thrombin in a 50 per cent glycerol solution is kept in the deep freeze and in such a solvent it will remain stable for several months. It is made up to a strength of at least 1,200 units per ml. and preferably more. This is buffered with 5 per cent imidazole buffer at pH 7 2 to 7.4 in a concentration of 12.5 per cent by volume of the solution Thrombin in weaker solutions of glycerol, such as 25 per cent is also stable but will freeze when placed in the deep freeze and the thawing process may reduce the strength. Refrigerator temperatures will maintain thrombin in glycerol solutions but not to the extent that the deep freeze will

(b) *Plasma* Plasma to be used is collected as described for all the other plasmas, a two syringe technique with the usual oxalate or citrate anticoagulants This plasma can be handled with more freedom than those in which Ac-globulin or prothrombin are to be measured because the antithrombin is more stable, but the usual care will be rewarded by more accurate work and it is advised to maintain the same careful standards for all Antithrombin will withstand several freezings and thawings as well as standing at room temperature for sometime It will remain at the same level in plasma for a few months in the deep freeze and seems to be one of the more stable clotting components When it is desired to do antithrombin studies the plasma must be defibrinated to remove the effect (antithrombin I) of the adsorption of thrombin on fibrin Also thrombin is to be added to this plasma and if there were any fibrinogen present a clot would form that would invalidate the test In order to accomplish this, heat is used to defibrinate, the plasma being carefully heated at 56°C for 3 minutes The heat coagulated fibrinogen can then be centri-

fuged to the bottom and the supernatant plasma pipetted off for use. Light centrifugation is all that is necessary; 1,000 g for 5 minutes will be sufficient. This heating does not interfere with the antithrombin effect, although higher temperatures may. If heating is not desired, the plasma may be defibrinated by the addition of a small amount of thrombin, as is done for the defibrination of plasma for a prothrombin determination. Apparently the addition of 0.1 ml. of a solution containing 100 units per ml thrombin, to 0.5 ml. of plasma will not materially affect the antithrombin determination. A comparison of analyses after the two methods of defibrination will reveal the same values.

(c) *Serum.* This is gathered as described in the accompanying directions for the reagents Since this serum should have no fibrinogen it is not necessary to defibrinate it. A slight amount of hemolysis does no harm.

Glassware. In order to maintain the strength of all thrombin solutions used in the manipulations it is necessary to use coated glassware. This may be done with paraffin or silicone. Any of the commercial products applied according to the manufacturer's directions seems suitable. Thrombin is adsorbed on glass and this is an important variable when dealing with the weaker solutions When stronger solutions, such as 1,200 to 1,400 units per ml , are handled the coating may be omitted, since the adsorbing quality of the glass will be exhausted quickly by these powerful solutions, and the percent adsorbed is low

Nevertheless, when these analyses are performed, coated glassware is used throughout to eliminate this action Re-use of siliconized glassware after washing is suspect and is not advisable Paraffin lined tubes can be prepared easily and are satisfactory for repeat use Any break in the continuity of the lining can be seen The determination of the final clotting endpoint does not require coated tubes

Steps in the Procedure This consists of allowing the specimen to react with a measured amount of thrombin for a set period of time and, at the conclusion of this time, measuring the number of units of thrombin remaining The plasma is first heat defibrinated as described before One ml is commonly heated and then 0.5 ml. of this is pipetted into a siliconized or paraffin lined tube after the fibrinogen has been spun down An equivalent volume of thrombin is added to the tube and the two are allowed to remain for two hours at room temperature At the end of this time, the tubes are placed in an ice bath, to slow the reaction until the amount of thrombin remaining is determined by means of the routine thrombin determination.

Therefore, where

T = standard concentration of thrombin

t = thrombin concentration in reaction mixture at the final equilibrium

then (a) = thrombin units destroyed by 1 ml. of plasma = (T) − 2(t)
and (b) = percentage of thrombin destroyed per ml. of oxalated plasma

$$= \frac{(T) - 2(t) \times 100}{T}$$

The answer is expressed as the percentage figure determined by the last equation. For the assay of thrombin the routine thrombin analysis described by Seegers and Smith is followed. This consists of the dilution of the mixture until a standard solution of fibrinogen is clotted in 15 seconds by the amount of thrombin contained therein (see page 184).

Precautions. The selection of the two hour time period for inactivation of thrombin is based on the fact that in the first 60 minutes of the reaction the destruction of the thrombin is progressive, becoming gradually less until an equilibrium is reached between the formation and destruction of the thrombin. This equilibrium has been observed in a large number of plasmas and always has been reached before two hours. Under these conditions of balance, the thrombin will be stable for a long time

The amount of thrombin in the standard solution is varied with the different specimens to be tested, a smaller amount for the smaller amount of antithrombin believed to be present, and the reverse for the stronger samples. If human plasmas are to be used routinely, a standard solution of about 1,400 units per ml. can be stored and used with satisfactory results These relationships between amounts of material used and inactivation are shown in table 1.

With each group of specimens a blank is run at the same time, using saline instead of plasma to mix with the thrombin At the end of the two hours there should be no destruction of thrombin in this system, and it can be used to check the test In addition to the blank run with the specimen, a normal plasma of the same species should be examined concurrently. This additional determination serves as a control on the activity of the reagents

TABLE 1 *Comparison of Potential Thrombin Inactivating Capacity of Serum and Defibrinated Plasma*

Units Thrombin in Substrate	Units Thrombin destroyed by 1 cc		Difference
	Plasma	Serum	
200	195	130	65
300	280	165	115
500	400	220	180
800	455	220	235
1100	375	165	210
1450	250	115	135

Serum· When serum is used the procedure is the same. It can be seen from table 1 that there is little less antithrombin activity in serum than in plasma. Of course, as stated before, there is no defibrination with serum and the process is thus simplified by the omission of this step.

In either of the analyses, on serum or plasma, it is advisable to run at least three determinations of the end point. These should check within a fraction of a second of each other, to be sure that an equilibrium has been established both as regards the antithrombin-thrombin system and also the dilution of the thrombin in the analysis

References

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9. *Thrombin Time*

J. C. PEDEN, JR. *and* G. BRECHER

The method is designed to measure heparin or heparin-like substances in plasma, although it is not specific for it. The test has been used empirically as an indicator of parenchymatous liver disease,[1] but has also given abnormal values in a number of unrelated conditions[2] The test has found its greatest usefulness in patients who have been heparinized as in cardiac surgery with extracorporeal circulation and hemodialysis, and in whom heparin is neutralized by a heparin antagonist at the end of the procedure Serial determinations of the thrombin time permit controlled administration of the antagonist until complete neutralization is achieved[3]

Apparatus Thirty-seven °C, water bath, 0 1 and 0.2 ml. pipettes, commercial bovine thrombin, toluidine blue.

Solutions

Toluidine blue 0.1 per cent solution in distilled water.

Thrombin stock solution 1000 units of thrombin are dissolved in 2 5 ml saline and then 2 5 ml. glycerol are added to give a *concentration*

then (a) = thrombin units destroyed by 1 ml. of plasma = (T) − 2(t)

and (b) = percentage of thrombin destroyed per ml. of oxalated plasma

$$= \frac{(T) - 2(t) \times 100}{T}$$

The answer is expressed as the percentage figure determined by the last equation. For the assay of thrombin the routine thrombin analysis described by Seegers and Smith is followed. This consists of the dilution of the mixture until a standard solution of fibrinogen is clotted in 15 seconds by the amount of thrombin contained therein (see page 184).

Precautions. The selection of the two hour time period for inactivation of thrombin is based on the fact that in the first 60 minutes of the reaction the destruction of the thrombin is progressive, becoming gradually less until an equilibrium is reached between the formation and destruction of the thrombin This equilibrium has been observed in a large number of plasmas and always has been reached before two hours. Under these conditions of balance, the thrombin will be stable for a long time.

The amount of thrombin in the standard solution is varied with the different specimens to be tested; a smaller amount for the smaller amount of antithrombin believed to be present, and the reverse for the stronger samples. If human plasmas are to be used routinely, a standard solution of about 1,400 units per ml can be stored and used with satisfactory results. These relationships between amounts of material used and inactivation are shown in table 1.

With each group of specimens a blank is run at the same time, using saline instead of plasma to mix with the thrombin At the end of the two hours there should be no destruction of thrombin in this system, and it can be used to check the test In addition to the blank run with the specimen, a normal plasma of the same species should be examined concurrently. This additional determination serves as a control on the activity of the reagents.

TABLE 1 *Comparison of Potential Thrombin Inactivating Capacity of Serum and Defibrinated Plasma*

Units Thrombin in Substrate	Units Thrombin destroyed by 1 cc		Difference
	Plasma	Serum	
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300	280	165	115
500	400	220	180
800	455	220	235
1100	375	165	210
1450	250	115	135

(2) The dilute thrombin solution is very unstable. It must be used soon after it is prepared.

(3) Glassware must be scrupulously clean.

(4) Some experience must be acquired in reading the end point in this test before reliable results can be obtained.

(5) Needles, syringes, and tubes used in this test must be absolutely free from any trace of heparin. The slightest contamination with heparin will result in extreme prolongation of the thrombin time.

References

[1] Harrington, W. J., Manheimer, R. J., Desforges, J. R., Minkel, H. P., Crow, C. B., and Stohlman, F. The bleeding tendency in hepatocellular and obstructive jaundice Bull New England Med Center 12 121-128, 1950

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10. Estimation of the Thrombin Time of Plasma

K. N. von KAULLA and E. von KAULLA

Objective of the Test and General Considerations. The plasma thrombin time measures the speed by which fibrinogen contained in the plasma is converted into fibrin by a standardized amount of thrombin

The test results depend upon substances with antithrombin II activity, on antithrombin V and VI, and, within certain limits, the fibrinogen content of the plasma They are independent from the activity of antithrombin III

Estimation of the thrombin time is important in research with various antithrombins or thrombin inhibitors The test is of clinical importance in obtaining immediate information on the effectiveness of heparin neutralization after extracorporeal circulation, for monitoring the waxing and waning of the occasional spontaneous appearance of thrombin-inhibiting substances during surgery, with cerebral and pulmonary embolism, after mismatched transfusions, and in other stressful situations With incoagulable blood, the thrombin time (in combination with the euglobulin lysis time) permits a rapid differentiation between afibrinogenemia, excessive thrombin inhibition, or a combination of both.

Principle Standardized amounts of thrombin are added to citrated plasma and the resulting clotting time is measured The goal is to determine

of 200 thrombin units per ml. This stock solution is stable for many weeks at 0°C.

Normal plasma is used as reference.

Performance of Test. Blood is collected in citrate anticoagulant. The blood is centrifuged and the plasma removed. A sample of normal blood is collected along with the unknown.

The stock solution of thrombin is diluted 1:100. This is the working thrombin solution containing approximately 2 units per ml

All reagents and tubes are kept at 37°C in a constant temperature water bath. Two-tenths ml of normal plasma is placed in a clean, unscratched 13 × 100 mm test tube and 0.1 ml of distilled water is added To this mixture is added 0.1 ml. of the working thrombin solution A stop watch is started upon the addition of the thrombin The contents of the tube are observed against a dark background with frequent tilting. When definite fibrin strands are first seen, the stop watch is stopped This dilute thrombin solution should give a thrombin time of 28-31 seconds with normal plasma. If results are shorter, the working solution must be suitably diluted with saline. If results are too long, a new working solution must be prepared from stock.

When a thrombin solution which gives an acceptable clotting time with control plasma is obtained, it must be used within a short time or it will rapidly lose strength

With the adjusted thrombin solution, control plasmas are re-run and their clotting times recorded Next, the patient's plasma is run exactly as the controls and results recorded (0 2 ml plasma, 0 1 ml distilled water, 0.1 ml thrombin solution)

If the thrombin time of the patient's plasma is prolonged, the toluidine blue solution is substituted for the distilled water in the test, i e , 0 1 ml. of [illegible] Both the patient's plasma and [illegible] l [illegible] na [illegible] he thrombin time of normal plasma with toluidine blue added, it is concluded that the prolonged plasma thrombin time was due to residual heparin and not to any of the other factors that may influence the test.[3]

Precautions

(1) The test must be carried out promptly after blood is drawn and the plasma separated The loss of CO_2, and the change in pH which will take place as the plasma stands will artefactually lengthen the thrombin time

(2) The dilute thrombin solution is very unstable. It must be used soon after it is prepared.

(3) Glassware must be scrupulously clean.

(4) Some experience must be acquired in reading the end point in this test before reliable results can be obtained.

(5) Needles, syringes, and tubes used in this test must be absolutely free from any trace of heparin. The slightest contamination with heparin will result in extreme prolongation of the thrombin time.

References

[1] Harrington, W J, Manheimer, R. J, Desforges, J R, Minkel, H. P, Crow, C. B, and Stohlman, F The bleeding tendency in hepatocellular and obstructive jaundice. Bull. New England Med Center 12 121-128, 1950

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Estimation of the thrombin time is important in research with various antithrombins or thrombin inhibitors The test is of clinical importance in obtaining immediate information on the effectiveness of heparin neutralization after extracorporeal circulation, for monitoring the waxing and waning of the occasional spontaneous appearance of thrombin-inhibiting substances during surgery, with cerebral and pulmonary embolism, after mismatched transfusions, and in other stressful situations With incoagulable blood, the thrombin time (in combination with the euglobulin lysis time) permits a rapid differentiation between afibrinogenemia, excessive thrombin inhibition, or a combination of both

Principle Standardized amounts of thrombin are added to citrated plasma and the resulting clotting time is measured The goal is to determine

the total of activities contained in the plasma interfering with the thrombin-fibrinogen reaction. The natural relationship of clotting factors, particularly of inhibitors and activators, should not be altered. Consequently, the plasma is not to be defibrinated, heated, adsorbed, diluted, or over-centrifuged to remove platelets (except when removal of platelets is specifically desired). Citrated plasma is used, because it is more sensitive than oxalated plasma in the detection of heparin and heparinoid material, though not overly sensitive as is EDTA plasma.

Reagents and Apparatus

Equipment:

(1) Five ml. and 10 ml. syringes with straight tip (B-D) multifit, preferably siliconized;

(2) 20-gauge needles (disposable 20 g x 1½ B-D #1000);

(3) 12 ml. heavy wall centrifuge tubes (glass, Corning #8120);

(4) 37°C. water bath (3 gallon all-glass fishtank; 500 Watt heating element coiled to fit the contours of the bottom; Brownwill contact thermometer 0-100°C. with two scales; Labline relay #3210), rack for suspending tubes in bath;

(5) 10 × 75 mm glass tubes with rim (Corning #9800);

(6) 1 ml graduated serologic pipettes (Kimble #37033); 0.1 ml. graduated serologic pipettes (Kimble #37036A);

(7) micropipettes, 5 lambda (Microchem Spec. # MG 005);

(8) stop watch,

(9) 250 beaker or paper cup with shredded ice,

(10) 100 ml beaker for flushing buffered saline through pipettes;

(11) 12 ml polypropylene tubes (Nalgene #3110);

(12) soft tissue (Kleenex)

Cleaning of glassware. Pipettes are cleaned with acid using the usual procedure.

Test tubes are cleaned with castile soap solution as follows. The clots are removed from the tubes shortly after the completion of the test and the tubes brushed with a rotating brush under running tap water The tubes are then allowed to soak in Castile soap solution for a minimum of several hours. Using a new soap solution, the tubes are cleaned for 20 minutes in the ultrasonic Sonblaster tank followed by thorough rinsing with running tap water, demineralized distilled water, and finally they are dried in an oven

Test tubes should not be cleaned with acid which produces, with repeated use, a surface comparable to that of siliconized glassware. Detergents are not recommended because they are difficult to remove from tubes even with extensive rinsing, thus interfering with the test.

Reagents

(1) Sodium citrate USP, 3.8 per cent solution in H_2O;

(2) Buffered saline, pH 7.4;

(3) Thrombin Parke Davis, 1000 NIH units (expiring date not less than 2 years from purchase date), dissolved in 5 ml. 50 per cent glycerol and stored at −15 to −20°C.

Buffered saline:

Stock barbital acetate reagent: Dissolve 9.714 Gm. $CH_3COONa \times 3H_2O$ and 14 714 Gm. Na-barbital in CO_2-free (boiled) H_2O. Make up to 500 ml.

Barbital acetate buffer, pH 7.42: Mix 5 ml. stock barbital acetate reagent; 5 ml. HCl 1/10 N; 2 ml. NaCl 8.5 per cent; 15 ml. distilled water. If minor pH adjustments are required, use stock barbital acetate reagent for shifting to the alkaline side, HCl for the acid side.

Working solution of buffered saline: 1 part barbital acetate buffer, pH 7 42 and 4 parts NaCl 0 85 per cent Mix well, check pH, and adjust if necessary with 1/10 n NaOH to 7.42.

Steps in Performance of the Test

Drawing blood

(1) Withdraw 2 ml sodium citrate into a 10 ml syringe, remove needle

(2) Using a fresh 20 gauge needle and a dry 5 ml syringe, perform venipuncture (avoid long stasis), withdrawing approximately 3 ml blood

(3) Leaving needle secure, remove syringe and attach the 10 ml. syringe containing the sodium citrate solution Draw blood exactly to the 10 ml mark.

(4) Withdraw needle and syringe from venepuncture site, remove needle from syringe, pull barrel back another ml and mix blood with citrate by tilting back and forth several times

(5) Discharge mixed blood gently without foaming into a 12 ml centrifuge tube and centrifuge for 5 minutes at full speed (3100 rpm or 1470 g) in an International Clincal Centrifuge Model CL (head #215 and four 15 ml carriers)

Test procedure

Into each of six glass tubes place 0.2 ml. citrated plasma and prewarm them in a water bath for approximately 3 minutes. Deliver 0.8 ml. buffered saline (cold from the refrigerator) into a polypropylene tube and place it in a beaker filled with shredded ice. (From now on one must work quickly

order to preserve the activity of the thrombin solution) Mix 0.05 ml. of ombin solution by blowing out (in order to mix well instantly) of a 0.1 . pipette into the buffered saline-containing tube. For the following test, : this once thrombin-contaminated pipette. Rinse it quickly with buffered ine, which is kept in a small beaker; wipe off. Take from this thrombin ution with the mentioned 0.1 ml. pipette, 0.1 ml. and blow into the first)e, start the stop watch at the same instant, and determine the appearance the first sign of a clot (tilting method). This is a practically instantane- s global clotting of the plasma with the shorter thrombin times and the pearance of a "hump" or of fibrin strands with the considerably pro- ıged times. Repeat with the second tube (these two tubes serve as plicates). This amount of thrombin used is referred to as thrombin di- ion A. Take 0.05 ml. of the thrombin-buffered saline mixture and blow o the third tube with plasma, start the stop watch; repeat with the fourth)e. This amount refers to thrombin dilution B Then take 0.025 ml. ombin solution (always with the same pipette, rinsed with buffered ine, blown out and wiped off) and blow into the fifth tube, and repeat :h the sixth tube. This refers to thrombin dilution C.

Should the pipette become clogged, use a fresh one, but rinse first with ombin solution and afterwards with buffered saline to "condition" it e pipette should be wiped off with tissue each time before introducing nto plasma. It is not necessary to run a normal plasma with the patient's ısma, because this test gives consistent results provided no inactivated ombin solutions are used.

TABLE 1 *Expression of Results*

:ombin dilution	A	B	C
ɔe	1 (and 2)	3 (and 4)	5 (and 6)
sma	0 2 ml	0 2 ml	0 2 ml
ombin-buffered saline solution	0 1 ml.	0 05 ml	0 025 ml.
its thrombin/ml plasma	5 85	2 92	1 46
rmal range of thrombin time (seconds)	7–9	9–12	14–16

'erpretation of Results

(a) *Thrombin time as such* A minor increase in antithrombin activity ects only dilution C With increasing activity, the lower dilutions will be)re and more affected Therefore it is important to record the results with three thrombin dilutions In human beings under certain pathologic con- ıons (e g, circulatory dysfunctions during physical or surgical stress), : thrombin time may exhibit rapid fluctuations Here serial determina- ns are much more revealing than one single test

(b) *Incoagulability of plasma* In extreme situations no thrombin time obtained at all, i e, the plasma remains incoagulable. This might be due

to an excessive titer of inhibitor, absence of fibrinogen, or both. The differentiation is achieved by the following two tests, c and d

(c) *Thrombin time in mixtures with patient's plasma and norm plasma.* Prolongation of the thrombin time in any dilution of mixtures co taining the patient's plasma and normal plasma above the normal plasn control indicates the presence of a high titer of a substance with an thrombin activity in the patient's plasma This test does not indicate tl presence or absence of fibrinogen in the patient's plasma The patien plasma (PP) is mixed immediately before the test with normal plasn (NP). The mixtures consist of 80 per cent PP + 20 per cent NP; 60 p cent PP + 40 per cent NP; 40 per cent PP + 60 per cent NP; 20 per ce PP + 80 per cent NP. Normal controls: 80 per cent buffered saline (BS + 20 per cent NP; 60 per cent BS + 40 per cent NP The titer of inhibit may be roughly estimated by the mixture with the highest of normal plasn content which exhibits prolonged thrombin time It should be noted th this test should only be used with very marked prolongation of the thromb times (dilution A above 45 seconds), particularly when the questic of inhibitor *versus* afibrinogenemia or severe hypofibrinogenemia arise Moderate prolongation of the thrombin time will not affect normal plasm

(d) *Euglobulin fraction* The presence of fibrinogen in the plasn sample incoagulable by thrombin is demonstrated by the coagulability of tl euglobulin fraction prepared from this sample With this test, most of tl thrombin-inhibiting substances are removed by the precipitation of tl euglobulin fraction The time required for spontaneous dissolution of tl thrombin-clotted euglobulins serves as a sensitive indicator for fibrinolyt activity (euglobulin lysis time)

(e) *Detection of heparinoid material* Correction of a prolonged plasn thrombin time upon addition of protamine sulfate or polybrene is suggestiv of the presence of heparin or heparinoid material neutralizable by the compounds It is recommended that one checks with a protamine sulfa range from 0 5 to 5 μg /ml plasma [This corresponds to an addition (0 001 to 0 01 ml. of a 0 01 per cent protamine sulfate solution to 0 2 m plasma Use a micropipette.]

(f) *Effect of $CaCl_2$* Often, addition of $CaCl_2$ (0 02 ml. 0 5 M CaC to 0 2 ml plasma) before adding thrombin normalizes more or less con pletely prolonged thrombin times with all thrombin dilutions In certa conditions the reduction of the thrombin time by $CaCl_2$ is missing or poo Marked prolongations of thrombin times which are poorly corrected t $CaCl_2$ appear to be related to hemorrhage.

(g) *Antithrombin V* The presence of antithrombin V can be suspecte to be the cause of the prolonged thrombin time when pathologic globuli

are simultaneously present. Antithrombin V is not corrected by protamine sulfate.

(h) *Antithrombin VI.* The presence of antithrombin VI can be suspected to be the cause of the prolonged thrombin time when other tests done simultaneously or directly preceding the thrombin time demonstrate a very marked increase of fibrinolytic activity. Antithrombin VI is only erratically corrected by protamine sulfate.

Storage. Plasma designated for thrombin time estimations can be kept deep-frozen for several days. Prolonged incubation or storage at room temperature should be avoided because the various materials with antithrombin activity have a different sensitivity to this type of storage.

Precautions and Sources of Error. The main source of error is the rapid inactivation of the working thrombin solution due to adsorption of thrombin to surfaces. This is particularly pronounced with glass. The use of polypropylene tubes reduces this danger, but even so, one individual batch of working thrombin solution should be used for one plasma only in one series of six to eight individual tests done in rapid succession. It is not recommended that one make up a greater amount of thrombin solution, because the more often the pipette is dipped in, the greater becomes the contact with the glass surface. The solution kept in ice water must be used immediately after its preparation.

The fibrinogen concentration of the test sample has some bearing on the results. A concentration below 100 mg per cent and above approximately 600 mg. per cent may prolong the thrombin time

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II. The Determination of Plasma Antithrombin Activity*

G. F. GRANNIS, L. A. KAZAL and L. M. TOCANTINS

Object of Test: To quantitatively assess the antithrombin activity of plasma.

Principle: When defibrinogenated, citrated plasma is incubated with a known amount of thrombin, the thrombin activity disappears according to first order reaction kinetics. The rate constant of the reaction is directly proportional to the amount of antithrombin in the incubation mixture and therefore can be related to the antithrombin activity of whole plasma.

Apparatus: (a) Small plastic test tubes (12 × 75 mm.), (b) large glass test tubes (to contain at least 10 ml.), (c) glass rods, prepared in 16 mm. lengths from O.D. 4 mm glass rod or tubing, (d) stop watch, (e) ultraviolet spectrophotometer.

Reagents: (a) 0 014 M sodium citrate, (b) exactly 0.25 per cent fibrinogen in 0 014 M sodium citrate, (c) 40 per cent urea in 0 2 N sodium hydroxide, (d) 0.15 M sodium chloride, (e) thrombin, 20 U./ml. (Parke, Davis & Co), in 0.014 M sodium citrate.

Steps in Performance of the Test

1 One-half ml of platelet-poor citrated plasma is defibrinogenated by adding 0 05 ml. of thrombin solution (20 U /ml. and incubating at 25°C for 10 minutes. The clot is removed by wrapping onto a glass rod

2 One and one-half ml. of 0 25 per cent fibrinogen solution is pipetted into each of seven small plastic test tubes in a 25°C. water bath.

3 Five ml of alkaline urea solution is measured into each of seven large test tubes.

4 Sixty-five hundredths ml of 0 014 M sodium citrate and 0.2 ml thrombin solution (20 U /ml.) are pipetted into a small plastic test tube in a 25°C water bath Fifteen hundredths ml defibrinated plasma is added, the contents are mixed thoroughly, and the stop watch is started

5 At appropriate time intervals (table 1), 0 10 ml aliquots are transferred from the incubation mixture to the tubes of fibrinogen and the solutions are mixed by gentle inversions

6 After exactly 14 minutes incubation at 25°C, a glass rod is inserted into the fibrin clot and the clot is wrapped onto it The rod with adhering

* Supported in part by Grant No H3544 from the National Heart Institute, N I H., U S P H S, Bethesda, Md

fibrin film is immersed briefly in a tube of 0.15 M sodium chloride to remove excess fibrinogen and is placed in a large test tube containing 5 ml. of alkaline urea solution.

7. When solution of the fibrin is complete, the rod is rinsed with 2 ml of 0.014 M sodium citrate, and set aside.

8. The absorbance of each fibrin solution is measured in a 1 cm. cell in the spectrophotometer at 282 mμ using a mixture of 5 ml. of urea solution and 2 ml. of 0 014 M sodium citrate as the blank

Calculation of Results The absorbance of each urea solution of fibrin is converted to thrombin units by means of a standard conversion table as previously described[1] The initial (4 minutes) determination is taken as 100 per cent of the available thrombin and all other values are calculated as per cent of the initial thrombin Per cent of initial thrombin is plotted on semi-log paper against the time of incubation of plasma with thrombin, and a straight line is drawn through the determined points (fig. 1).

The observed rate constant of antithrombin activity, k, is calculated from the relation

$$k = \frac{2.3\ (\text{log per cent initial thrombin} - \text{log per cent thrombin remaining at time t})}{t}$$

or, selecting the time at which half of the initial thrombin is utilized:

$$k = \frac{2\ 3\ (\log 100 - \log 50)}{t_{1/2}} = \frac{0\ 693}{t_{1/2}}$$

in which $t_{1/2}$ is the time at which 50 per cent of the initial thrombin was consumed A unit of antithrombin activity is defined as that amount which causes thrombin to disappear at the rate of 0 01/minute Hence the units of antithrombin (A U) in the sample assayed is.

$$\text{A.U} = \frac{k}{0\ 01} = 100\ k$$

The determination as described is performed in an incubation mixture which is 13 64 per cent plasma Since the rate constant of antithrombin activity is directly proportional to antithrombin concentration (fig 2), the antithrombin activity of 100 per cent plasma is.

$$\text{A U of whole plasma} = \frac{100\ k}{0\ 1364} = \frac{100}{0\ 1364} \times \frac{0\ 693}{t_{1/2}} = \frac{508}{t_{1/2}}$$

Normal Range of Values In a series of 25 normal plasmas, antithrombin values of 29 to 42 units per ml of plasma (mean 34) were observed Increases of 4 to 20 times normal are observed among patient plasmas

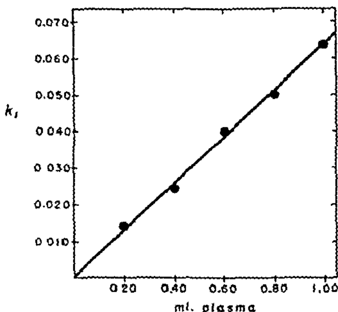


FIG 1—The first order plot of plasma antithrombin activity Defibrinogenated plasma was incubated with a standard thrombin solution, and thrombin was determined at intervals

Precautions and Sources of Error Plasma antithrombin activity has been observed in this laboratory to follow first order reaction kinetics over the pH range 6 5 to 10 0 and at salt concentrations ranging from 0 01 M to 2 0 M The pH activity curve of plasma antithrombin is bell-shaped with maximum activity occurring at pH 8 5, and in going from pH 7 0 to 8 5 a four-fold increase in activity is observed In the analytical procedure described, antithrombin is determined at pH 7 4 and low ionic strength, and care should be exercised that all plasmas are compared at the same pH

Occasionally results are obtained which do not give a linear plot, suggestive of a decline in antithrombin activity during the course of the determination However, this seems to be due to the addition of excessive amounts of thrombin to the plasma, when these determinations are repeated using lesser amounts of thrombin, linear plots of results are obtained It is recommended that the total amount of thrombin added to plasma (both for defibrination and as substrate for antithrombin) should be within the range observed during the thrombin activity curve [7]

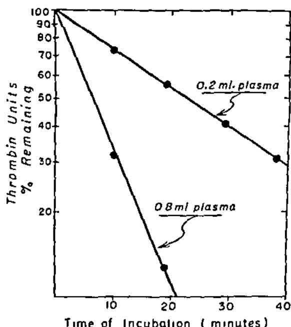


Fig 2.—The linear relation of the observed rate constant of antithrombin activity to the concentration of plasma antithrombin in the incubation mixture

Table 1. *Time Schedule of Operations for Performing Four Antithrombin Determinations Simultaneously*

Plasma No	Start Reaction	Remove Aliquot from Incubation Mixture					Remove Fibrin Clot				
	(time in minutes)										
		a	*b*	*c*	*d*	*e*	*a*	*b*	*c*	*d*	*e*
I	0	4	8	12	16	20*	18*	22*	26	30	34
II	1	5	9	13	17	21*	19*	23*	27	31	35
III	2	6	10	14	18*	22*	20*	24	28	32	36
IV	3	7	11	15	19*	23*	21*	25	29	33	37

* [illegible]

minutes

Reference

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12. Estimation of Heparin in Blood after Injection[1]

L. B. JAQUES

Heparin, while not normally present in the blood, appears in it under certain pathologic conditions (peptone shock, anaphylactic shock) and is present in the blood of subjects who have been receiving the drug. The following method may be used to deproteinize plasma for assay of heparin.

Reagents: Sodium citrate 3 8 per cent, ether, phenol solution. Phenol is liquefied in a water bath and 800 ml. are diluted to one liter with distilled water.

Procedure: Nine ml. of blood are drawn and added to 1 ml. of sodium citrate solution and centrifuged at 2,000 rpm for ten minutes. The plasma is pipetted off and the cells are washed with 1 ml. of 0 85 per cent NaCl. This wash is added to the plasma and the cells are discarded. Phenol solution, 5 5 ml., is added, the tube is corked and the phenol and plasma are thoroughly mixed by vigorous shaking. The tube is allowed to stand at room temperature for ten to twelve hours, after which it is centrifuged at 2,500 rpm for 20 minutes. The clear upper layer is pipetted off carefully and the phenol layer washed with a small amount of 0 85 per cent NaCl. The combined aqueous phase is washed with 5 ml. of ether The ether layer is pipetted off and the ether remaining in solution is removed by vacuum or by heating in a water bath for a minute or two at 65°C. The solution may be then assayed by the methods described on page 383 For routine or clinical work the method can be shortened by omitting the two washings. It has been found that even with this latter change, highly reproducible recoveries (80 per cent) can be obtained by this procedure For smaller or larger volumes of blood, quantities of saline, phenol, etc., are taken in the given proportion An earlier, alternative method is described by Jaques, Monkhouse and Stewart[2]

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13. The Ultracentrifugal Separation of Lipoproteins for Coagulation Studies (Method of Kazal, Miller and Tocantins)*

L. A. KAZAL and L. M. TOCANTINS

Object: To separate alpha-1, alpha-2 (+chylomicra) and beta lipoproteins from human plasma by preparative ultracentrifugal flotation in a form suitable for inclusion in blood clotting tests, primarily for anticoagulant studies of the interaction of lipoproteins with phospholipids

Principle. The lipoproteins are separated at 4°C. from citrated plasma by successive ultracentrifugations; first at the native density (d) of the citrate plasma which separates by flotation the alpha-2 lipoproteins and the chylomicron fraction at the top of the centrifuge tube; then, at densities of 1.063 and 1.21, which separate by flotation the beta and alpha-1 classes of lipoprotein, respectively The separated fractions are dialyzed against a citrate-saline solution and concentrated to a standard volume The basic method is that of Gofman and co-workers[1] as modified by Bragdon et al.,[2] except that plasma is used instead of serum, fractions are separated in a citrate-anticoagulant solution (equivalent to citrate plasma), and the lipoproteins are dialyzed and ultrafiltered to provide concentrates

Reagents· (1) Human plasma collected in 19 per cent sodium citrate; (2) citrate-saline solution, pH 7.2· 0.85 per cent NaCl + 0.022 M sodium citrate; (3) diluent solutions· citrate saline at d = 1.63 and at d = 1.21, adjusted with NaBr. All chemicals are reagent grade

Apparatus· (1) Siliconized syringes, 10, 20, and 50 ml capacity; (2) silicone-coated Baxter TransfusoVac bottle, (3) International PR-2 Centrifuge for 1800 g, (4) 50 ml Lusteroid centrifuge tubes, (5) Spinco Model L Preparative Ultracentrifuge with No. 40 rotor and 12 aluminum-capped plastic tubes of 11 5 ml capacity; (6) hydrometers, specific gravity ranges 1.000-1.200 and 1 200-1 400; (7) glass cylinder, 100 ml, silicone-coated for specific gravity measurements, (8) cellophane dialyzing tubing, 1" and 1⅝" flat width, (9) special stainless steel syringe needles, B 16 gauge, with 3½" long stem and blunt end (cut-off) (modified needle has the last ¼" curved to 45° angle for plasma collection), a similar curved 20 gauge needle, 2¾" long, for ultracentrifugal fractions, (10) beakers, 50, 250 and 600 ml. capacity, polypropylene, (11) pycnometer, 10 ml, (12) sharp-pointed scissors or razor blade

* Supported by Grant No H-3544 of the National Heart Institute, N I H, U S P H S, Bethesda, Md

General Description of Procedure: Each of the three lipoproteins is separated from plasma as a crude fraction and is purified by recentrifugation. This is accomplished in four ultracentrifugal runs as follows: *The first* involves the separation of the crude alpha-2 (chylomicron) fraction at the native density of plasma; *the second*, the purification of alpha-2 fraction and the separation of beta lipoproteins from subnatant plasma of the first centrifugation; *the third*, the purification of beta and of alpha-2 remnant volumes from above, and the separation of crude alpha-1 fraction from the subnatant plasma of the second centrifugation; and *the fourth*, the purification of alpha-1 lipoprotein and of any beta lipoprotein remnant volumes.

The following unit operations are employed for the separation of each fraction:

1. *Ultracentrifugations.* All ultracentrifugations of plasma or of lipoprotein fractions are conducted at 4°C. and 40,000 rpm for 24 hours in a Beckman Spinco Model L ultracentrifuge with a #40 rotor containing 12-11.5 ml plastic tubes with aluminum caps. Eleven ml of solution are placed in each tube, with this volume the layer of lipoprotein is visible and thus is more easily separated from the subnatant plasma. The total volume of plasma or solution that can be processed at one time is 132 ml. After centrifugation, both rotor and tubes must be handled carefully so as not to disturb the separated layer of lipoprotein. The collection of the top layers (lipoprotein fraction) should proceed promptly, since the centrifugation is conducted at 4°C and mixing by convection may occur at room temperature This is not experienced if the separations are made immediately after removing the cold rotor from the centrifuge.

2. *Density adjustments* The densities of the subnatant plasmas are adjusted to 1 063 (or 1 21) with NaBr Measurements are made with a pycnometer or, more conveniently, the measurement of specific gravity with a hydrometer is sufficiently accurate for this purpose A correction is made for the density due to protein This density is obtained by subtracting the electrolyte density (1 006) from the measured density of the plasma. The density due to protein then is added to 1 063 (or 1 21) to obtain the density at which the sample should be centrifuged The amount of NaBr required is estimated on the basis that 0 028 Gm of NaBr/ml changes density by 0 02 units The addition of NaBr is carried out in two steps· In the first, the amount to be added is so calculated as to approximate the desired density but not to equal or exceed it, in the second the additional amount required to give the desired density is added The calculations follow.

(1) Density of subnatant plasma −1.006 (density of electrolyte in citrated plasma) = density (protein increment).

(2) Density (protein increment) + 1.063 (or 1.21) = desired density.

3. *Collection of lipoprotein fractions:* The lipoproteins which accumulate in the top layer of the tube after centrifugation are removed by the "rimming" technic. The fraction is withdrawn at the surface of the solution with a 10 ml. syringe by simultaneously applying gentle suction and moving the 20 gauge, bent-tip, blunt needle along the inner circumferential edge of the tube. Caution must be exercised to avoid drawing in air bubbles and also to prevent inadvertent mixing of top layer with subnatant plasma, which occurs if any withdrawn solution is forced back accidentally into the tube The entire procedure is carried out with the aluminum cap on the tube

4. *Collection of subnatant plasma·* After removing the top layers, the subnatant plasma is best collected by cutting off the aluminum cap (scissors or razor) in order not to contaminate the subnatant plasma with a trace of top layer which clings to the cap.

5 *Overlaying operation·* Purification of collected top fractions is accomplished by flotation of the lipoprotein into a layer of solution at the appropriate density For this purpose, 5 5 ml of diluent consisting of citrate-saline solution adjusted with NaBr to the required density is placed in the tube and 5.5 ml of lipoprotein fraction is forced gently and slowly under it with a 10 ml syringe and a 4 inch-16 gauge straight blunt-end needle. The overlay solution thus is raised as a layer to the top of the tube without mixing it with the lipoprotein solution

6. *Dialysis* The high concentration of NaBr present in the lipoprotein fractions is removed by dialysis in Visking casing bags (1 inch flat diameter) at 4°C against a 200-fold volume of citrate-saline solution, with daily changes for 3 days

7 *Ultrafiltration* The dialyzed solutions are concentrated to a standard volume equivalent to 0 05 ml per ml of citrate plasma by ultrafiltration according to the centrifugal technic of Prasad and Flink[3] Approximately 7 ml of fraction is obtained Dialysis bags must be tested under pressure for any leaks before filling the fraction to be ultrafiltered

8. *Storage and Stability* Purified lipoprotein concentrates are stored over nitrogen in silicone-coated vials at 4°C

Preparation and Purification Procedure

1 *Collection of plasma* Human plasma is collected from fasting donors or patients in a silicone-coated Baxter bottle (300 ml. of blood) as described for the preparation of *fibrinogen*[4]

2 *First Ultracentrifugation· Separation of alpha-2 (+chylomicra) lipoprotein.** Ultracentrifugation of citrated plasma (d=1 006 ± 0.001) for 24 hours is sufficient to separate by flotation the chylomicron and alpha-2 lipoprotein fraction.† Eleven ml. of plasma are placed in each of 12 plastic tubes, capped and centrifuged. The lipoprotein fraction separates as a milky, white layer at the top of the tubes with a distinct clear zone of liquid below it. These layers are removed by the "rimming" technic, pooled and held for recentrifugation. The yield is variable: 1 to 3 ml. per tube.

The subnatant plasma in the tubes is collected in a 250 ml. beaker. The gelatinous protein sediment found in the bottom of the tube is dissolved in the plasma and requires some mixing with a glass rod. The subnatant plasma contains beta and alpha-1 lipoproteins, which are separated in the second centrifugation.

3 *Second ultracentrifugation·* a *Separation of beta lipoprotein at d = 1.063.* Nine tubes are reserved for this separation. The subnatant plasma from the previous centrifugation is adjusted to d = 1.063 with NaBr and, if necessary, the volume is increased to 99 ml. with diluent solution at d = 1 063 The tubes are filled, capped and placed in the pre-cooled rotor and centrifuged (with alpha-2 fraction, see 3b) for 24 hours. The orange-yellow top layer (3 ml) is collected, pooled and held for purification in the third centrifugation The d = 1 063 subnatant plasma is held for separation of alpha-1 lipoprotein

b. *Alpha-2 lipoprotein purification* The three tubes reserved for the purification will hold a total volume of 16 5 ml This is usually sufficient to purify the entire volume of crude alpha-2 fraction, if the yield is greater, the excess volume must be held over for purification in the third centrifugal run Purification is accomplished by overlaying 5.5 ml. of fraction with 5.5 ml of citrate saline solution (d = 1 006) in three tubes, and centrifuging (with the nine tubes of subnatant plasma from 3a) After centrifugation for 24 hours, the milky white layers are collected, pooled, dialyzed, ultrafiltered and stored at 4°C.

4 *Third ultracentrifugation·* a *Separation of alpha-1 fraction* The d = 1.063 subnatant from the previous centrifugation is pooled and the gelatinous sediment dissolved as in the previous run The density then is adjusted to 1 21 and the volume, if necessary, to 66 ml with diluent at d = 1 21 Six tubes are filled and centrifuged (with 4b) for 24 hours A

* Fraction designated as alpha-2 according to Kunkel and Trautment (J Clin Invest 35 641, 1956) based on electrophoretic migration on starch media

† The contribution of protein to the density of the solution is not considered because the bulk of the plasma protein separates from the system in the region where lipoproteins accumulate by flotation

yellow-orange fraction is found in the top layer with a very narrow and barely visible clear zone below it. The top layers are collected and held for purification in the fourth centrifugal run. The d = 1.21, subnatant plasma is held for dialysis.

b. *Purification of beta lipoprotein.* The pooled beta fraction from 3a is purified by overlaying in three tubes with diluent at d = 1.063· 5.5 ml. fraction per tube. (Any excess volume is held at 4°C for purification in the fourth run.) After centrifugation for 24 hours, the orange-yellow top layers are collected, dialyzed, ultrafiltered and stored at 4°C.

Comment. In the third centrifugation, three tubes are reserved for the *purification of any remnant volumes of alpha-2 fraction left over in 2.* Or, conversely, all of the beta fractions may be centrifuged here and the alpha-2 fraction held over for the fourth run If an odd number of tubes is obtained, the rotor is filled with tubes containing a diluent of appropriate density to provide proper ballast. This arrangement also is utilized in the fourth centrifugation

5 *Fourth ultracentrifugation:* a. *Purification of alpha-1 lipoprotein* The pooled top layers from 4a are filled into three tubes (5.5 ml per tube) and overlayed with diluent at d = 1.21 After centrifugation, 2 to 2.5 ml. of alpha-1 lipoprotein are collected from the top layer, dialyzed, ultrafiltered, sealed, capped and stored at 4°C. preferably under nitrogen.

b. *Purification of beta or alpha-2 lipoprotein.* Any remnant volumes of these fractions are centrifuged in this run and combined with their respective purified fractions

6 *Subnatant plasma d = 1 21·* The plasma remaining after the lipoprotein fractions are removed may be dialyzed exhaustively against 0 0002 M sodium citrate solution, lyophilized and restored with distilled water to provide a concentration of 7.5 per cent protein and 0 02 M sodium citrate

Comments and Precautions

1 *General procedure.* The procedure describes the separation of lipoproteins from 132 ml of plasma into three classes, each concentrated approximately 20-fold Smaller volumes may be ultracentrifuged, but the ultrafiltration procedure becomes more difficult to perform, as the yield decreases. The lipoproteins of a normal subject and of a patient may be separated simultaneously in one series of centrifugations without much additional labor by using six tubes for each The handling of one or two tubes of plasma is more difficult, but possible, and requires a modified approach to the adjustment of density Under these conditions, dilution of the plasma sample is greater, whereas in the described procedure for larger volumes only small amounts of diluent are necessary during centrifugation

The procedure is adaptable to the selection of other classes of lipoprotein For example, fractions from a series of densities at 1 006, 1.018, 1.063 and 1.21 have been obtained A larger number of fractions offers a greater degree of fractionation but increases an already long centrifugation schedule. On the other hand, less specific fractionation is also feasible and the procedure may be limited quite readily to one or two fractions at selected densities, but in this case complete separation of conventional classes of lipoprotein is not achieved. With a two-step centrifugation at d = 1.21, a product containing the lipoprotein classes of plasma combined in one fraction may be obtained.

2 *Dialysis*. Since concentrations of inorganic salts above 0.15 M show inhibitory effects on coagulation, dialysis to eliminate the high concentration of NaBr (approximately 24 Gm. per cent at d = 1.21) is essential. This is accomplished by using 0 85 per cent NaCl containing 0 022 M sodium citrate at pH 7 2 to provide an ionic strength equivalent to that present in the original citrate plasma The concentration of citrate in plasma is calculated with due allowance for a normal hematocrit and the volume of plasma trapped by the sedimented erythrocytes The use of citrate is beneficial in its capacity to complex metal ions through chelation, while not as active as ethylene-diamine tetracetic acid (EDTA), in this respect, nevertheless, it is sufficiently effective to prevent oxidation of lipoproteins by trace metal ion catalysis At the same time it supplies the anticoagulant originally present in the plasma, and simplifies the inclusion of these lipoprotein fractions into plasma clotting tests

3 *Ultrafiltration* It is desirable to reduce the lipoprotein fraction to a constant volume, 5 per cent of original plasma volume, so that the activities of normal subjects and patients may be compared Since lipoprotein concentration in plasma is variable anyway, and yields are low, it is more difficult to standardize by weight The volume technic offers a convenient point of departure for comparing lipoprotein activity by reference to a standard plasma level arbitrarily selected at 20 x plasma concentration If desired, lipoprotein concentration may be obtained from the total solids, determined by drying the lipoproteins at 100°C to constant weight and correcting for the salt concentration of solvent.

4 *Storage* Under nitrogen, activity is retained for 1 week and thereafter is reduced in an unpredictable fashion

Results. Lipoproteins prepared by this method have essentially the same physical and chemical properties as lipoproteins separated by ultracentrifugal flotation from serum With regard to blood coagulation, they have been shown to interact with phospholipids, particularly cephalin and phosphatidylserine [8] The end product of this interaction is a lipoprotein-

phospholipid complex which possesses an anticoagulant activity in recalcified plasma and in thromboplastin generation clotting tests. The results indicate that phospholipid micelles are solubilized by alpha-1 lipoprotein through a binding, or an interaction, with the lipoprotein The net effect of this interaction is the conversion of the phospholipid from an insoluble micelle exhibiting thromboplastic activity to a *soluble complex* exhibiting anticoagulant activity. Interaction of phospholipid with beta lipoprotein of certain patients results in the formation of an *insoluble complex* with anticoagulant activity that is considerably greater than the insoluble complex obtained from normal subjects which is inactive. The insoluble alpha-2 complex is usually inactive. Some observations with alpha-1 lipoprotein are illustrated in figure 1.

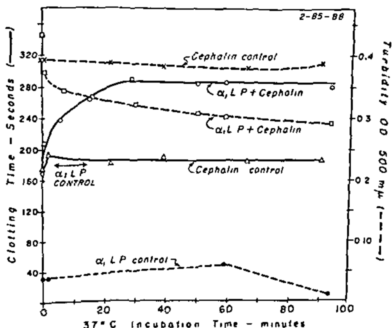


FIG. 1.—Solubilization of phospholipid by normal alpha-1 lipoprotein separated from human plasma at d = 1 21 Phosphatidylserine and cephalin complexes formed during incubation with lipoprotein solution at 37° C develop anticoagulant activity as measured by the cephalin clotting time Associated with the increased activity is the progressive reduction in turbidity (optical density) of the incubating mixture measured spectrophotometrically at 500 mμ Concentration of phospholipid in the clotting test mixture (anticephalin test) is *330 μg per ml The phospholipid concentration in the incubating mixture is* 880 μg per ml.

Preparation of lyophilized cephalin for lipoprotein studies A convenient, reproducible and stable suspension of cephalin, developed for long-term studies of the activity of lipoproteins, has been prepared by a modification of the method of Tocantins and Holburn (Chap XI, Section 3). Starting with 10 Gm quantities of acetone-dried brain powder, cephalin is precipitated eight times as described (p 301). The final ethanolic supernatant then is discarded and the precipitate transferred with anhydrous acetone to a tared 50 ml. beaker; the acetone is evaporated with N_2 gas at 37° C. The acetone-dried residue is further dried to constant weight (18 hours) in a vacuum oven attached to a freeze-dry apparatus A 0.2 per cent (w/v) suspension in 0 85 per cent NaCl is prepared with a tissue hand homogenizer. The suspension is divided into 5 ml aliquots in 10 ml vials (Wheaton & Co), plug-frozen in dry ice-acetone mixture, and lyophilized After 40 hours of lyophilization, the vials are capped with rubber stoppers and pressed-on aluminum seals Yields range from 3 to 5 per cent of dry weight of acetone-dried brain powder The physical properties and the activity of the original suspension has been maintained for as long as 4 years when the vials were stored at −8° C. to −20° C. When used, the product is restored with distilled water, heated to 60° C. for 20 minutes, and cooled to and held at 38° C in order to provide a suspension with unchangeable activity It may be reused on the 2nd day after heating to 38°C. for 10 minutes Both lyophilized and liquid products should be stored under nitrogen for maximum protection against oxidation The procedure for preserving lyophilized material under nitrogen is described on page 234, item 5

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CHAPTER XII

TISSUE COAGULANTS AND ANTICOAGULANTS

1. *Estimation of Thromboplastin Activity of Tissue Extracts*

W. H. SEEGERS

(a) In Vitro Two-Stage Method

The tissue material which apparently consists of lipoprotein has the property of accelerating the activation of prothrombin far more rapidly than any other known procoagulant This power is due to the fact that the two enzymes thrombin and autoprothrombin C come out of the prothrombin molecule and then activate the prothrombin This is the basic autocatalysis which dominates in all prothrombin activations. When tissue extracts (thromboplastin) are not used and lipid activator(s) (partial thromboplastin) are employed practically, only thrombin is the catalyst and very little autoprothrombin C is involved

In the well known prothrombin time determination the tissue extracts can be tested quite easily The more potent ones are likely to produce clotting in 12 seconds whereas with the weaker ones progressively longer clotting times are found according to the diminution of strength This approach to our assay can be very useful provided the concentration of prothrombin, autoprothrombin I, and Ac-globulin in the test plasma are normal. Any of these substances in the tissue extract will have a bearing on the result

Usually the material which is obtained by high speed centrifugation seems to be weak. This is because the washing removes substances that are important in the prothrombin activation when the one-stage test is employed The high molecular weight material has, however, a very high procoagulant power which can be demonstrated with the use of purified prothrombin as a substrate. In principle the prothrombin, Ac-globulin

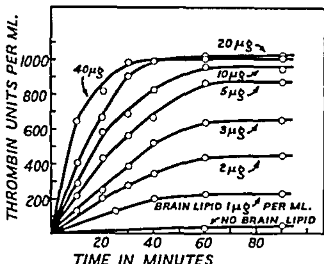


FIG 1.—The activation of prothrombin with use of brain thromboplastin, Ac-globulin and calcium ions at 28° C and pH 7 2 The activation mixture is described in the text The thromboplastin concentration is noted on each curve From data of Seegers, Cole and Aoki.[8]

and calcium ion concentrations are fixed If the conditions are properly arranged, practically no thrombin activity generates unless thromboplastin is also added The amount of thrombin which forms depends upon the amount of thromboplastin added The quantity of autoprothrombin C which forms also depends upon the amount of thromboplastin added. Since it is far more convenient to assay for the thrombin activity than the autoprothrombin C, the former is the one to measure

In a typical assay the following activation mixture was set up at pH 7 2 and 28°C

Purified prothrombin (3,000 U /ml)	1 part
Ac-globulin concentrate (300 U /ml)	1 part
Calcium ions (0 075 M)	1 part

From time to time, samples were taken to assay for thrombin activity Very little was generated Then the experiment was repeated several times with various amounts of sedimentable thromboplastin (brain thromboplastin, bovine) suspended in the calcium solution Samples were again taken for thrombin analysis A series of thrombin concentration curves was then plotted (fig 1). The thrombin yield increased with the strength of the procoagulant From such data it is possible to assign a value to the

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rial is then expressed as the number of MLD or "units" per ml Ordinarily about 8 mice will be used for the calibration of an unknown, after the preliminary determinations.

Light ether anesthesia may be used for convenience during the test apparently without disturbing the endpoint. The endpoint of the assay is the death of the mouse in a few minutes after the injection, usually within two minutes, and not more than five. Some animals may become comatose and convulse following the test, but recover. Such a reaction indicates that a near lethal dose has been given Almost always, the mice are able to throw off the effects rapidly if the dose is not fatal at once

Injection of the material must be intravenous The lethal dose is increased markedly if the injection is given slowly or in divided doses Backflow of blood into the needle or the barrel of the syringe should be avoided The syringe is filled with the exact dose and after the insertion of the needle the extract is forced into the vein in a single rapid injection

Usually, a suitable lethal volume is of the order of 0.10 ml, and in performing the assay, the extract is diluted to this activity The dose given to each animal may be increased or decreased by changing the volume of the extract to be injected, or by dilution of it, or both, according to the response of the animal, or animals, used in the test Experience is the most valuable guide to the initial dilution and volume A suggested maximum volume of any material is 0 4 ml The assay, under proper conditions, is reliable to approximately 10 per cent

A rapid tolerance develops in the animals after the injection of the thromboplastin The animals may be used again for "screening" assays, but they should not be used until after at least three days, preferably longer The increased tolerance of the animals to the toxin is at its highest within a few minutes and persists for a few hours, at the end of eight hours, the animals will have returned to nearly the previous level of sensitivity Similarly, complete "desensitization" can be established by the use of small repeated dosages.

The sensitivity of the mouse is not directly proportional to the body weight, large or obese mice being more sensitive per gram of body weight Corrections to the lethal dose for mice of 20 grams body weight can be made by comparison with standard curves if desired Unless the variation of weight is extreme, the error without correction, as compared with mice of 18 to 21 grams, is not great

The investigation of the sensitivity of the mouse has also brought out the fact that the animals become more sensitive as they become older and larger The optimum weight is about 18 to 21 grams. This represents the body weight of a young, healthy laboratory mouse.

potency of the thromboplastin preparation. The value can be expressed in thrombin units or these can be translated into arbitrary thromboplastin units.

For an assay, the strength of the procoagulant must be adjusted so that the thrombin yield is less than the total thrombin potential of the prothrombin substrate. If more than one dilution is studied, the information becomes valuable in characterizing the procoagulant power of the thromboplastin preparation. This test is sensitive to variations in Ac-globulin and autoprothrombin I, but not to an extent that readily invalidates results.

The Ac-globulin concentrate needed is made by the method of Aoki, Harmison and Seegers.[1] The prothrombin by the method of Seegers.[2] The assay for thrombin by the method of Seegers and Smith.[3, 4] For a detailed interpretation the work of Seegers, Cole and Aoki can be consulted.[5]

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(b) *In Vivo* Assay (Method of Schneider)

Young, white mice are used as the test animals Other experiments with different strains of white mice as well as brown mice and wild mice have shown that they are all suitable as test subjects No sex difference has been noted, except that during pregnancy the sensitivity increases

Trial doses of the extract to be tested are given intravenously in the lateral tail veins of weighed mice until the minimum lethal dose for a 20 gram mouse can be determined or estimated from interpolation curves which have been constructed previously in the testing laboratory by injection of the thromboplastin of different dilutions The toxicity of the mate-

erly evaluate thromboplastic activity in a PE preparation, the following questions must be carefully considered:

1. *Purity of the PE preparation.* Only one spot (corresponding to a standard PE) should be detectable when 200 μg of the intact phospholipid are chromatographed on paper impregnated with silicic acid (see p. 367). This would indicate that the quantity of possible contaminants (not detected by this method) would be approximately of the order of less than 1 per cent. PE fractions from various natural sources contain some aldehydogenic PE as well as the diester form

2. *Degree of oxidation.* The polyunsaturated fatty acids which occur in phosphatidylethanolamines from different natural sources are very vulnerable to oxidation by contact with oxygen from the air During the course of preparation of pure PE fractions, extreme care must be used to work under nitrogen whenever possible and the final product should be stored under nitrogen in a desiccator at −30 to −65°C. As oxidation proceeds, the PE darkens in color and it becomes more and more difficult to prepare suspensions with potent acceleratory activity from the oxidized phospholipid

3 *Preparation of suspensions of PE* Although exact criteria for obtaining a maximum number of micelles of PE with size and shape to yield optimal thromboplastic activity are not yet known, we have been able to produce suspensions of PE which at very low concentrations can replace platelets in thromboplastin-generating mixtures The procedures follow: A. *"Paste method"* The work is carried out under nitrogen whenever possible All suspensions and dilutions are made with buffered saline solution (BPSS) * The BPSS is flushed with nitrogen for 5 minutes before use. Approximately 10 mg of the PE fraction is weighed in a 50 ml. beaker. BPSS is added in increments of 0 2 ml The PE is crushed in the BPSS with a spatula after each addition of BPSS to make a homogeneous paste (no visible large particles) Finally, up to 6 ml of BPSS is added (1 ml at a time, mixing with a spatula after each addition) to produce a good suspension (no settling out of particles) The beaker contents are transferred to a 14 ml pyrex tube containing nitrogen and the beaker is rinsed with two 2 ml portions of BPSS which are transferred to the tube This produces a suspension of PE (1 mg /ml) of fairly large particle size Nitrogen is run over the surface of the solution to remove air and the tube is stoppered immediately with a clean rubber stopper The tube is then shaken vigorously by hand for 30 seconds This reduces the turbidity of the 1 mg /ml suspension A 20 μg /ml suspension is finally prepared by diluting 1 ml of the 1 mg /ml suspension with 49 ml BPSS B *Alter-*

* Nine tenths per cent NaCl solution buffered with 0 05 M imidazole, pH 7 4

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2. Evaluation in Vitro of Coagulant and Anticoagulant Activities of Phospholipids*

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Methods are presented for the evaluation of the coagulant activity of phosphatidylethanolamine (PE) and phosphatidlylserine (PS) and the anticoagulant activity of PS in in vitro clotting tests. It is hoped that this will provide orientation for those with little experience in this field and help to explain apparently conflicting reports in the literature. The main difficulties involved in the reliable assay of the activity of the phospholipids in blood clotting concerns their chemical purity (including knowledge of presence of contaminants in a fraction) and the nature of the colloidal state of the actual suspension or solution being tested. Although precise information is still not available concerning the optimal number, electric charge, size and shape of specific phospholipid micelles necessary for optimal coagulant or anticoagulant activity, good activities have been obtained by employing the preparations described herein

The material presented is based mainly on experience in the author's own laboratory. Important contributions to progress in this field have been made by many investigators whose work cannot be described here because of limitation of space

Phosphatidylethanolamine

Thromboplastic activity of phosphatidylethanolamine.

Phosphatidylethanolamines from such diverse sources as brain tissue, red cells, platelets, soy beans, rabbit appendix, eggs, yeasts and fungi and *synthetic di-oleoyl PE* have been reported to have thromboplastic activity [1] PE has not been reported to have anticoagulant activity In order to prop-

* This chapter is adapted from reference No. 4, to which the reader is referred for further details

clotting times which are shorter than those obtained in the second control test (no phospholipid present in the incubation mixture) at similar incubation times are indicative of an accelerating effect. Substrate clotting times longer than the corresponding control times are indicative of an inhibitory effect. Dilute suspensions of PE have been stored by us under nitrogen at −65°C. and have been found to be fully active when thawed and tested for thromboplastic activity up to 6 months later. Typical results for activity of such suspensions in the HPT are shown in figure 1.

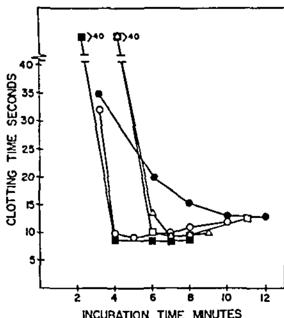


FIG 1—Activity of phosphatidylethanolamine suspensions in the Hicks-Pitney test. ■————■—Crude brain phospholipids 30 µg in incubation mixture Acceleratory control. Suspension by method A in text ●————●—No phospholipids added to incubation mixture Volume replaced by buffered saline solution O————O—Synthetic dioleoyl PE freshly prepared by Dr D L Turner (Cardeza Foundation, Jefferson Medical College, Philadelphia, Pa.) Fifty µg in incubation mixture Suspension by method B in text □————□—Purified PE fraction from beef brain (contains traces of other phospholipids (LPE, PS, IP) Two and one-half µg in incubation mixture Suspension by method A in text Note relatively strong activity of small amount of PE. Probably due to influence of trace contaminants on micelle size and shape of colloidal particles of PE △————△—Synthetic dioleoyl PE of Dr. E. Baer (University of Toronto, Canada) Fifty µg in incubation mixture. Fraction preserved for 4 years under nitrogen at −65° C Suspensions by method B in text

native procedure. Some PE preparations are very refractory to formation of good suspensions by procedure A. When this occurs, it has been found that such PE preparations may form active stable suspensions in solutions of sodium deoxycholate, probably by micelle formation with the deoxycholate The deoxycholate itself is inert in the clotting tests at the concentrations employed. The PE is solubilized by first adding 0.1 ml of sodium deoxycholate solution (1 mg./ml. in BPSS) to 10 mg. of PE in a 50 ml. beaker and crushing the PE in the solution with a narrow spatula to form a homogeneous paste. Increments of the deoxycholate solution are then added (at first 0.1 ml, then larger volumes) followed by similar crushing and mixing with the spatula. When complete dispersion of the PE is obtained (no large particles seen), the suspension is brought to a concentration of 1 mg./ml. by adding the requisite amount of deoxycholate solution. Test solutions are then prepared by diluting with BPSS to make solutions of 100 μg./ml of PE and deoxycholate as desired. C. *Methods involving ether.* Wallach, Maurice, Steele and Surgenor[3] have described a method for preparing suspensions of PE with thromboplastic activity by injecting a concentrated ethereal solution of the PE into the requisite amount of final diluent and then removing the ether. This is similar to a method employed by Silver et al [2] to prepare solutions of phosphatidylserine. We have also combined this method with method B by injecting the ethereal solution into deoxycholate solution and then removing the ether We prefer to use these methods only when method A or B fails, since some ether is no doubt incorporated into the phospholipid micelle and little is known about such ether-containing micelles.

Testing for thromboplastic activity.

The dilute suspensions described above and dilutions of them are used for studying thromboplastic activity by substituting them for a platelet suspension in the Hicks-Pitney Test (HPT) The HPT is performed exactly as described on p. 94 except that the plasma is centrifuged for 60 minutes at 3000 rpm. Two controls should always be run at the beginning and end of each series of tests First, an acceleratory control is run to show that the system is working well This should have a suspension of platelets, mixed phospholipids or a PE fraction in the incubation mixture. When the system is working properly, substrate clotting times should be 11 seconds or less after incubation for 5 minutes or less The second control consists of an incubation mixture with no added phospholipid, i e, diluted plasma plus BPSS After about 10 minutes incubation, substrate clotting times come down to about 15 to 25 seconds and level off under these conditions This lower level can vary somewhat depending on the particular plasma employed. For use as a research tool we consider that all substrate

at the concentrations employed, then inhibitory activity can be demonstrated in the tests already mentioned and the antithromboplastin test (see p 300) Kind of micelles obtained and particle size no doubt vary, depending on the exact conditions employed in preparation of dispersions or solutions of PS and these in turn will influence activity in in vitro clotting tests Figure 2 shows differences in clotting activity in suspensions and solutions of PS prepared in different ways and tested at similar concentrations.

Preparation of suspensions of PS. These can be prepared by the "paste" method as described under 3A in the section on phosphatidylethanolamine Usually an adequate stable suspension can be prepared *without* the shaking described there

Preparation of solutions of PS: A. *With sodium deoxycholate* A solution of sodium deoxycholate (Matheson, Coleman and Bell) at 2 mg /ml in BPSS (pH 7.4) is first prepared. One-tenth ml of the solvent is then added to 10 mg. of solid PS in a small beaker or test tube After grinding and crushing with a narrow blade spatula for a few seconds, a homogeneous paste is formed. Addition of a few tenths ml more of solvent produces a good suspension A clear solution at a concentration of 2 mg /ml. PS can then be made by bringing the total volume to 5 ml with larger increments of solvent Dilution of this PS solution can then be made using BPSS as diluent. This keeps the ratio of PS to sodium deoxycholate at 1/1 (w/w) B. *With albumins* Clear solutions of PS can be made by simply adding an albumin solution (albumin 10 mg /ml in distilled and deionized water) to solid PS to give a final concentration of PS of 1 mg /ml Dilutions of this PS solution can then be made using water as the diluent The ratio of PS to albumin is thus held at 1/10 (w/w) Such solutions have been successfully prepared using human, bovine, porcine, equine and rabbit albumins They have anticoagulant activity [4]

3. *Interaction of phosphatidylserine with other substances* It is known that phospholipids may interact with each other and with various classes of substances which are normally present in blood A *Effects of inorganic salts.* That PS can bind sodium and potassium ions has been shown by studies on uptake of radioactive K and Na In our laboratories we have observed the precipitation of PS from solution by $HgCl_2$ and $CaCl_2$ solution A PS solution with anticoagulant activity in the HPT, if treated with $CaCl_2$ solution, will form a suspension with accelerating activity On the other hand, if the PS solution is added to plasma first and then the $CaCl_2$ solution is added (as is routinely done in clotting tests), no precipitation occurs Apparently interaction between PS and some plasma constituent(s) interferes with precipitation by $CaCl_2$ solution B *Bile salts* PS can be well solubilized by sodium deoxycholate and to a lesser extent by other bile salts [3] This is probably due to formation of an association

For the present we prefer to work in clotting systems employing citrated plasma only, considering this to be minimal departure from physiologic conditions. However, Wallach et al.[2] have employed a modified thrombin generation test using fractions of factors V and VIII along with Ca^{++} and Mg^{++} and a suspension of PE to convert prothrombin (purified) to thrombin which is measured on fibrinogen substrate. Such tests have revealed important information concerning the relationship of the colloidal state of PE to thromboplastic activity and no doubt will be used more extensively in the future.

Phosphatidylserine

Activity in blood clotting of phosphatidylserine fractions

This phospholipid has been reported to have both inhibitory and acceleratory activity in in vitro clotting tests and anticoagulant activity when given intravenously to dogs.[1,3] In evaluating the activity of a PS fraction in coagulation it is essential to bear in mind the following considerations:

1. *Purity of the PS fraction* As for PE, the minimum criterion for purity should be the detection of one spot corresponding to PS when 200 μg of the phospholipid are chromatographed on paper impregnated with silicic acid (p 368) On storage, most PS fractions originally pure by this criterion show traces of a second component, lysophosphatidylserine (LPS) and/or oxidized PS This generally causes enhancement of anticoagulant activity of PS fractions, probably by aiding in solubilization of PS. Although precautions are taken whenever possible to minimize oxidation of PS during its preparation, this is not as critical (at least for anticoagulant activity of PS from pork and beef brain) as for PE. PS from these sources probably has considerably smaller amounts of conjugated polyunsaturated fatty acids than PE. It is oxidized at a much slower rate and the partially oxidized fractions appear to maintain their anticoagulant activity. PS fractions isolated from pork and beef brain all give a positive Schiff's test and probably contain small amounts of aldehydogenic PS as well as the diester.

2 *Solubilization of PS fractions* Experience in the preparation of suspensions and solutions of PS for evaluation of inhibitory or acceleratory activity in coagulation has convinced us of the importance of particle size and, no doubt, micelle size and shape and electric charge as determining factors in kind (inhibitory or acceleratory) and degree of activity of PS When *pure* PS is dispersed in BPSS, the resulting suspension can be shown to have acceleratory activity in the thromboplastin generation test, the HPT and the plasma recalcification test However, if the PS is solubilized to form clear solutions by interaction with naturally occurring substances such as albumin and bile salts, which in themselves are inactive

colloid with the deoxycholate. This solubilization will take place in the presence of 0.9 per cent sodium chloride. Sodium deoxycholate dissolved in BPSS is actually used for solubilizing the PS The resulting clear solutions have anticoagulant activity. C. *Other phospholipids.* Although interaction of PS with other phospholipids has not yet been studied extensively, it is known that under certain conditions (in chloroform solution) an interaction product between PS and lecithin can be obtained which (after the removal of the $CHCl_3$ and suspension in BPSS) has clot-accelerating activity. Data, as yet unpublished, has been accumulating in our laboratory indicating that when LPS (and perhaps oxidized PS) is present in some fractions, they are partially solubilized and show weak inhibitory activity in the recalcification and antithromboplastin tests without addition of other solubilizing agents Ordinarily, simple *suspensions* of pure PS show acceleratory activity at low concentrations in the Hicks-Pitney and plasma recalcification tests It is quite possible that PS may interact with other phospholipids D. *Mucopolysaccharides.* It has been shown that PS can interact with at least one mucopolysaccharide which can influence its activity in blood clotting The inhibitory activity of very small amounts of solubilized PS can be strongly enhanced by amounts of heparin which have negligible inhibitory activity in the clotting tests employed. Other mucopolysaccharides have not as yet been investigated. E *Proteins* The interaction of PS and albumins to form clear solutions with anticoagulant activity has already been mentioned Interaction of PS and other plasma protein fractions is currently under investigation We have already found that while Cohn fraction IV-1 and simple suspensions of PS have acceleratory activity in the recalcification test, mixtures of these two have inhibitory activity Also IV-4 alone neither accelerates nor inhibits, but when mixed with a suspension of PS, inhibitory activity is obtained. Fibrinogen fractions have also been shown to interact with PS to form clear solutions with inhibitory activity Plasma protein fractions are now being systematically investigated by us

Since all of these types of substances do occur in plasma, it is obvious that the addition of a phospholipid like PS to plasma in a clotting test results in a very complex situation with all the above possibilities for interaction operating simultaneously First of all, interaction between PS and the substances mentioned above can produce interaction products of different micellar size and shape with the possibility that chemical groups active (either acceleratory or inhibitory) in clotting may be oriented toward the center of the micelle or outwardly in the surrounding milieu and this may be a factor in determining their activity Elucidation of this situation will no doubt be forthcoming after detailed studies in which efforts are made to correlate different biophysical and biochemical parameters with activity in

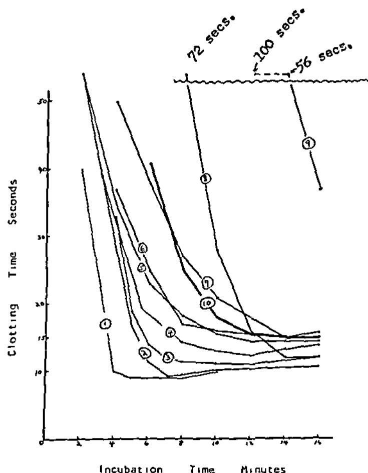


FIG. 2.—Effect of Phosphatidylserine (beef brain fraction) in the modified Hicks-Pitney Test. Results show influence of the colloidal state of the suspension or solution of PS on clotting activity The fraction employed showed one spot (PS) by paper chromatography. Suspensions showed varying degrees of turbidity, solutions were water clear. Curve 1 is an acceleratory control (30 μg of mixed brain phospholipids in the incubation mixture) to show that the system is functioning well Curve 10 is a control with no phospholipids present in the system The suspension and solutions were prepared as described in test Curve 1 mixed brain phospholipids —30 μg; curve 2. PS suspended in BPSS, (ether method)—5 μg, curve 3 PS suspended in BPSS, (paste method)—5 μg, curve 4 PS suspended in PSS with no buffer, (paste method)—5 μg.; curve 5: PS suspended in distilled water, (paste method)—5 μg; curve 6· PS suspended in imidazole buffer with PH 7 4, (paste method)—5 μg; curve 7 PS dissolved in human albumin solution—5 μg, curve 8 PS dissolved in sodium dexycholate solution—5 μg; curve 9: PS dissolved in sodium deoxycholate solution—10 μg; curve 10 control—no phospholipid in incubation mix

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3. Preparation of Phosphatides for Use in Studies of Blood Coagulation

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Reagents

Glacial acetic acid, analytical reagent (A.R.)

Acetone, A.R.

B.D.H. Universal indicator (The Ealing Corp., Cambridge, Mass. agent for the British Drug Houses, Ltd., Poole, Great Britain)

Cellulose powder, Whatman. (This must be thoroughly washed, as described by Rouser et al.[1])

Chloroform A.R. This should be distilled and then stabilized by the addition of 1 per cent methanol. It should be tested for acidity by means of a benzene solution of Rhodamine 6 G, and if the indicator changes color to red, the chloroform should be discarded.[2]

Diethylaminoethyl cellulose ("Cellex D", Bio-Rad Laboratories, Richmond, Calif.)

Ethanol, absolute. Do not use denatured alcohol, even when dry it changes the solvent distributions.

2, 4-Lutidine (85 per cent technical (Distillation Products Industries, Rochester, N.Y.). This should be distilled before use.

Ninhydrin (Pierce Chemical Co., Rockford, Ill.). If ninhydrin does not work well, it should be purified as described by Hamilton and Ortiz.[3]

Petroleum ether, b.p. 30-60°C., Merck, "Suitable for fat determination."

Silicic acid, Merck reagent (Catalog No. 71503, Merck & Co., Rahway, N.J.)

coagulation. Secondly, since PS can interact with so many substances present in plasma, the resulting activity in coagulation must depend on the nature of the summation of effects produced by competition for sites of "interaction" or "binding" on the PS molecule among the various substances with which PS can "interact." This in turn will be regulated by the *specific experimental conditions in the clotting tests* employed, the purity of the PS fraction tested and the reagents and methods employed for dispersing or solubilization of the phospholipid.

Testing for acceleratory effect on suspensions of PS in coagulation: We employ two tests for assessing the clot-accelerating effect of *suspensions* of PS.

A. Hicks-Pitney test. This is performed as already described for PE.

B. Recalcification test. To 0.1 ml. of human plasma (upper ¾ of supernatant from citrated blood centrifuged at 3000 rpm at 4°C. for 1 hour) in a siliconized clotting tube (14 x 58 mm) at 37°C are added 0.1 ml. of test solution or control and 0.1 ml of $CaCl_2$ solution (0.02 M). A stop watch is started. The end point is the first visible sign of clot formation.

Testing for inhibitory effect of solutions of PS on coagulation: The anticoagulant effect of solutions of PS is tested in three tests.

A. Hicks-Pitney test. Performed as described for PE.

B. Recalcification test. Performed as described in B above. Some typical results are shown in table 1.

C. Antithromboplastin test. To 0.1 ml of human plasma (prepared as in B above) in a siliconized clotting tube at 37°C. are added 0.1 ml test solution or control, 0.1 ml brain thromboplastin, 0.1 ml $CaCl_2$ solution (0.02 M), with mixing after each addition. The stop watch is started on addition of the $CaCl_2$. The end point is the first sign of clot formation. Some typical results are shown in table 1.

TABLE 1 *Inhibitory Effect of Solutions of Beef Brain Phosphatidylserine on Coagulation PS dissolved in albumin*

µg PS in Clotting Test	Antithromboplastin Test* Clot Time (seconds)	Recalcification Test* Clot Time (seconds)
0	20	320
1	25	480
2	31	720
5	36	1080
10	58	2000
25	72	no clot overnight
50	90	" " "
100	130	" " "

* See text for clotting tests employed

quickly attached to a trap cooled in dry ice by means of an adapter containing a stopcock. The stopcock is necessary in order to admit nitrogen after the drying process is complete. The trap is evacuated by a rotary oil pump

Source of Phosphatidylethanolamine for Use as an Accelerator in Blood Clotting Studies

It is not easy to find a suitable source of phosphatidylethanolamine. The reason for this is apparent from the important studies of Rouser, White and Schloredt[10,11] and of Wallach, Maurice, Steele and Surgenor.[12] The highest activity appears in preparations containing the greatest amount of unsaturated fatty acids, and it is also important that the acids should be unsaturated in both the α and β position of the glycerol moiety. It has also been shown by Rouser that the plasmalogen form of phosphatidylethanolamine has no activity Unfortunately, some of the sources richest in phosphatides with unsaturated acid radicles also contain the most plasmalogen In addition, the highly unsaturated materials are extraordinarily susceptible to oxidation

These difficulties can be avoided by using preparations made by total synthesis. The simplest synthesis of unsaturated phosphatidylethanolamines is that described in detail by Daemen, de Haas, and van Deenen[13] It has been shown by Turner, Holburn, deSipin, Silver, and Tocantins[14] that synthetic racemic dioleoylphosphatidylethanolamine is as active as platelets in the Hicks-Pitney test, but not as active in the thromboplastin generation test. This paper also summarizes the earlier work of Rouser, Kuhn, and others on this subject.

A simple preparation of a representative sample of the total phosphatidylethanolamine of egg is described here This material is active in the Hicks-Pitney test. It can form a starting point for the isolation of more unsaturated preparations by following the methods of Wallach, Maurice, Steele and Surgenor.[12] The method of preparation is an application of technics introduced by Rouser.[5] This method is also suitable for the purification of phosphatidylethanolamine from other sources However, for preparations other than egg, the loading of the column should be reduced Rouser recommends a load of no more than 350 mg for columns of 15 Gm of DEAE cellulose acetate We have isolated active phosphatidylethanolamine from mushrooms by this technic, but their iodine numbers were about 100 It is possible that a preparation of higher iodine number could be made from soy bean phosphatide The phosphatidylethanolamine of soy bean has been shown by Rouser and Schloredt to be very active[11]

Technic of Paper Chromatography[4,5]

Strips of Whatmann filter paper No. 3MM are impregnated with silicic acid as described by Marinetti[5] Samples of 300 μg. are applied, and the papers are used as described by Marinetti, in pyrex cylinders containing diisobutyl ketone-acetic acid-water (40:20:5) at 5°C. in a cold room. The staining of the papers is described by Marinetti;[5] the most useful stains for the phosphatides described here are Rhodamine 6 G, followed by ninhydrin.

Preparation of the DEAE Cellulose (Adapted from Rouser[1]*)*

A quantity of about 100 Gm. of Cellex D is put into a 4-liter beaker and stirred with distilled water. After settling, the supernatant water is decanted together with the fines. This process is continued until the fines are removed, (about eight washings). The material is then stirred with 2 liters of 1 N hydrochloric acid for 10 minutes and filtered with suction on a Buchner funnel. It is washed in the funnel with several liters of water and then transferred to a beaker and washed with water by decantation until the washings are completely neutral The material is then converted to the free base with 1 N sodium hydroxide solution in a similar manner and washed until neutral It is filtered and sucked dry on the Buchner funnel It is dried in air, and then covered with glacial acetic acid. After stirring, the mixture is allowed to stand for 3 hours. The product is filtered, sucked dry, and stirred again with acetic acid and allowed to stand overnight. It is filtered as before, washed twice with methanol on the funnel, and then transferred to a round-bottomed flask This is attached to a trap immersed in dry ice attached to a high vacuum rotary oil pump The acetic acid remaining in the material is sucked off for 2 days The DEAE cellulose acetate is then transferred to a bottle for storage It should be used within two weeks

(a) *Apparatus for evacuation under nitrogen* This is described in reference 6.

(b) *Apparatus for extraction of tissue* This consists of a three-necked round-bottomed flask of a volume suitable for the amount of solvent In the central opening, a stirrer of the Hershberg type[7] is inserted through a sealed bearing The bearing is described in the Organic Chemical Bulletin of Eastman Kodak Co[8] The other two openings of the flask have an entry and exit tube for nitrogen

(c) *Apparatus for filtration* Filtration was performed by sucking off the solution through a filter thimble as described in reference[9]

(d) *Apparatus for freeze-drying.* The material to be dried is frozen in a shell in a 1-liter round bottomed flask with a ground joint and this is

from entering the eluate. The DEAE cellulose acetate is introduced in methanol above the cellulose powder. The material in the column is washed with 2 liters of methanol, or more, until the eluate gives a negative test for acetic acid* with the B.D.H. indicator. The column is washed with 600 ml. of chloroform and, as the last chloroform runs into the DEAE cellulose acetate, up to 3 Gm. of the fraction is introduced in chloroform and allowed to run into the column, fresh chloroform being used to wash in the fraction. Care must be taken not to let the column run dry. The top of the column is attached to apparatus (a) and nitrogen is introduced. The mixture is then eluted with chloroform as long as material is eluted as indicated by evaporation of a portion of the effluent. The chloroform (A) can be discarded Then the column is eluted with 4 per cent methanol in chloroform (v/v), and (B) then with 8 per cent methanol in chloroform (C) and finally 12.5 per cent methanol in chloroform (D), the volumes now used being controlled by the ninhydrin test on the effluent

In conducting this elution, it is possible to run the solvent through at a speed of up to 15 ml per minute It is convenient to observe the first appearance of ninhydrin-positive material by taking a few drops of effluent and heating with a drop of 0 3 per cent ninhydrin in n-butyl alcohol and a drop of lutidine, as described by Rouser et al.[1 16] The composition of the fractions is examined by paper chromatography Usually, A contains fat and lecithin, B is pure lecithin, C is a mixture of lecithin and PE and D is pure PE A little ninhydrin-positive material runs into B at the beginning and can be segregated by the ninhydrin test If pure PE is not obtained from the evaporation of fractions C and D, the fractions containing PE are chromatographed again on a fresh column of DEAE cellulose acetate The evaporation of the solvents is performed as described previously and the products are stored in a freezer under acetone and nitrogen.

Characteristics of Egg PE

The PE obtained from four egg yolks with DEAE cellulose acetate amounts to more than 500 mg and represents the total PE of the egg It has an iodine No of 100

Wallach, Maurice, Steele, and Surgenor[14] purify egg PE on columns of silicic acid These columns are slow, have very low capacity and only small quantities can be prepared However, the PE can be fractionated on silicic acid to give material with a much higher iodine No than the average PE of egg It has been reported[11 12] that the highly unsaturated PE is a

* The test was "negative" when the color of the indicator was similar to the color produced by an identical volume of 0 0005 N acetic acid The mixture of methanol and indicator must be diluted with water to obtain the true color

Preparation of Phosphatidylethanolamine (PE) from Hens' Eggs

The initial stage of this preparation is adapted from Wallach, Maurice, Steele and Surgenor.[12] Hen's egg is a desirable starting material because it lacks phosphatidylserine. The yolks of four eggs are separated and rolled gently on cloth to remove adhering albumin They are then washed into a flask with a little water and freeze-dried. The dry residue (about 30 Gm) is then transferred to the extraction flask (b) together with 250 ml. of acetone. A stream of nitrogen is introduced and the stirrer started. After thorough mixing, the stirrer is removed without interrupting the nitrogen and quickly replaced with the filter apparatus (c). The filter tube is attached to a suction flask and evacuated through the apparatus (a). To complete the filtration, the nitrogen tubes are replaced with rubber stoppers, nitrogen is introduced through the filter flask and filter thimble and then the suction is continued to remove as much acetone as possible. The system is again filled with nitrogen through the filter thimble, and 250 ml. of acetone is added. The stirring apparatus and nitrogen tubes (b) are quickly put on again. After stirring under nitrogen as before, the acetone is again sucked off The two acetone extracts are discarded. Then an extraction with 250 ml of chloroform-methanol 2·1 (v/v) is performed by stirring under nitrogen, and this is sucked off into a second filter flask A second extract is made with a fresh 250 ml of chloroform-methanol 2·1. The two chloroform methanol extracts are combined under nitrogen in a separatory funnel. After adding 100 ml of 1 per cent potassium chloride solution, the funnel is filled with nitrogen, shaken, stoppered and the layers are allowed to separate The lower layer is then washed again in the funnel with 100 ml of the equilibrium upper layer of Folch,[15] consisting of chloroform-methanol water (3.48:47) The lower layer is drawn off into the edge of a conical funnel containing three filter papers. The funnel is covered by a larger funnel, through which nitrogen is passed The three filter papers suffice to dry the extract The solvent is evaporated in vacuo at 30°C in a rotating evaporator filled with nitrogen before evacuation, using the apparatus (a) The residue is stored under 200 ml. of acetone in a flask filled with nitrogen at −25°C

Separation of Lecithin and PE on a Column of DEAE Cellulose Acetate

A quantity of 50 Gm of the DEAE cellulose acetate is put in a 1-liter beaker and stirred with methanol It is then filtered in a Buchner funnel and put back in the beaker. A column of the type used by Rouser et al ,[1] 40 mm. in diameter, is used The bottom of the column has an irregularly shaped glass ball on which a layer of glass wool is placed. Then a suspension of Whatman cellulose powder in methanol is poured into the column to form a layer 4 inches deep. This is for the purpose of preventing DEAE cellulose

The second ether treatment described by Folch is conducted in the same manner using one-quarter as much ether.

The precipitation of the "cephalin" is also conducted in centrifuge bottles and the "cephalin" precipitate is collected by centrifuging rather than filtration as used by Folch.

The collection of the Folch fractions I, II, III is also performed under nitrogen by centrifuging. The precipitation of the "cephalin" and the fractions I, II, III, is performed at room temperature.

Fraction III contains most of the PS The yield from 1500 Gm. of brain is 3 5 to 5.7 Gm. Losses are usually caused by transfer of PS into fractions I and II. This can be recovered by doing the Folch separation in chloroform ethanol to get a second fraction III treating the I and II as starting material.

Paper chromatography shows both fractions III to be PS and inositol-phosphatide (IP) and traces of PE. Dialysis is not used

Preparation of Pure PS from the Folch Fraction III

Chloroform and methanol for the further purification of Folch fraction III are precooled to 4°C. *The material is dissolved in the cold chloroform* using 12 ml. per Gm

To the solution, add the cold methanol with swirling using 22 ml per Gm The operation is conducted in centrifuge bottles The bottles are filled with nitrogen, stoppered, and centrifuged for 20 minutes at 4°C. and 2000 rpm. This gives a viscous liquid underlayer. The supernatant is *decanted* (III_1) *The precipitate is treated with cold chloroform and* methanol exactly as before, using the original volumes In this manner, fractions III_1, III_2 and III_3, III_4, III_5, and III_6, etc are collected as long as appreciable material is extracted The final insoluble material is III_R Fractions excluding III_1, III_2 and III_R are pooled, provided paper chromatography shows them to be pure PS. The soluble fractions from III_4-III_8 are always pure PS Often, *fraction III_3 is PS also* The other fractions contain PS with traces of contaminants, IP in III_R, PE, phosphatidic acid and lysophosphatides in III_1 and III_2 The total yield of pure PS is from one-third to one-half of the fraction III.

(b) *Preparation of PS from pork brain by the method of Rouser*[1] *(modified)* This is an easier method for obtaining PS, if it is only needed in small quantities. This PS is free acid, whereas the preparation obtained by the Folch method is a mixture of sodium and potassium salts

Fresh pork brain (195 Gm) is ground in a Waring blender with 1300 ml. of acetone pre-cooled to −20°C The mixture is transferred to the three-necked extraction flask under nitrogen The solvent is sucked off The brain is then extracted 3 times with 800 ml portions of acetone under nitrogen, the mixture being stirred for 10 minutes in each extraction. The

particularly potent thromboplastic agent. The average PE described here is fully thromboplastic when properly solubilized and is equivalent to platelets in the Hicks-Pitney test.[14]

The PE obtained from the DEAE cellulose columns sometimes contain small traces of lecithin. It forms a convenient starting material for additional purification on columns of silicic acid, if this is required. The most convenient method is that described by Barkhan, Silver, and O'Keefe.[17]

Separation of phosphatidylethanolamine on columns of silicic acid[17] Silicic acid is separated by screening, and material of 40-100 mesh is selected. This is then washed thoroughly with methanol and chloroform by decantation with rejection of fines.

A column is prepared in chloroform as described above using silicic acid (150 Gm.) in place of DEAE cellulose acetate. To this column, apply 1.5 Gm. of crude egg phosphatide or material purified partly on one column of DEAE cellulose acetate. The column is then eluted in succession with chloroform, 5 per cent methanol in chloroform and then with 10 per cent methanol in chloroform. As soon as the effluent gives a blue color with the ninhydrin test, the receiver is changed and 15 per cent methanol in chloroform is run through as long as a positive test is obtained (about 2 liters). This is the phosphatidylethanolamine fraction. Evaporation of the solvents gives 200 mg. or more of phosphatidylethanolamine. Pure lecithin can be removed from this column with 50 per cent methanol in chloroform. The flow rate is 7-8 ml. per minute.

Preparation of Phosphatidylserine (PS) From Beef Brain

(a) *Method of Folch,*[17] *modified by Turner, Silver and Tocantins*[19]: The procedure starts with the preparation of Folch fraction III and follows Folch in every detail Care is taken to operate under nitrogen wherever this is feasible. The brain is extracted immediately after removal from the slaughterhouse. It should never be stored In the process of removing membranes and blood vessels, most of the white matter is also lost. Quantities of 1500 Gm of brain are used at one time If it is necessary to store the tissue overnight, this should be combined with the extraction process and the extraction continued under nitrogen It is possible to reach the first petroleum ether extraction in 1 day and then leave the tissue in petroleum ether overnight All evaporations are conducted at 35°C in vacuo under nitrogen The crude extract, after removal of the petroleum ether, is transferred with ethyl ether to 250 ml centrifuge bottles, with 12 Gm in each bottle The bottles are filled with nitrogen and stored in the cold room for precipitation of the cerebrosides For each Gm of extract, use 15 ml of ether. After standing overnight at 40°C, the cerebrosides are centrifuged out at 2700 r p m. at 4°C. for 30 minutes.

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brain residue is then extracted 4 times with 500 ml portions of chloroform-methanol, 2:1 by volume. The extracts are sucked off and preserved under nitrogen. The total chloroform-methanol extract is shaken under nitrogen in a separatory funnel with ⅕ volume of 1 per cent potassium chloride (Folch wash procedure).

The lower layer is filtered under nitrogen through three filter papers to dry it. It is then evaporated below 35°C. in vacuo under nitrogen. The residue is shaken under nitrogen with 250 ml. of acetone precooled to −10°C. The acetone is discarded and the insoluble material is taken up in dry ether and transferred to a centrifuge bottle. A total of 70 ml. of ether is used The solution is centrifuged into two centrifuge bottles, and treated with 5 volumes of ethanol. The mixture is centrifuged at room temperature. The insoluble material, weighing about 1.8 Gm. has the composition of a "cephalin" (PS, inositolphosphatide and PE) The soluble material is mostly lecithin and PE

A column of 30 Gm. of DEAE cellulose acetate is prepared in methanol and washed with methanol until neutral. It is then washed with chloroform A quantity of 360 mg of the "cephalin" is applied in chloroform and the column is then washed with chloroform, chloroform-methanol 7:1 and chloroform-methanol 3:2 as long as these solvents elute material The column is then eluted with pure methanol, 500 ml., and 25 per cent acetic acid in chloroform, 500 ml These fractions are discarded. The PS is removed from the column with glacial acetic acid, only the fraction giving a pure blue test with ninhydrin being collected The acetic acid is removed by freeze-drying leaving a pure PS (190 mg) All work is done under nitrogen

While probably not necessary, it is our practice in this laboratory to filter such material through a column of cellulose powder as described by Rouser et al [1]

Total synthesis of PS The saturated PS synthesized by Baer and others is too insoluble for testing in clotting systems. A racemic dioleoyl-PS has been synthesized in this laboratory from diolein, phosphorus oxychloride and carbo-*tert*-butyloxy-dl-serine phthalimidomethyl ester It shows activity in clotting systems similar to the activity of natural PS [20]

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initial "wetness" of the tissue as well as its consistency. The above procedure is repeated until a fine grayish white powder is obtained This is then spread on a large, dry, flat piece of glass and the residual acetone removed by evaporation. This process is hastened somewhat by frequent changing of the exposed surface by means of a spatula, constantly spreading and tamping. Excessive exposure to air during the drying process should be avoided The dried powder is then passed through a #20 mesh wire screen and is ready for further processing.

Extraction· To 20 grams of the acetone dried powder previously described is added 800 ml. C. P. absolute methanol and the mixture allowed to remain in a wide vessel at 5°C. for five days, with occasional shaking. The supernatant solution is filtered through two thicknesses of Whatman #2 filter paper and the filtrate distilled *in vacuo* in a flask immersed in an H_2O bath maintained at 45°C. The brownish residue remaining in the distilling flask is removed with absolute ethyl ether, the ether solution then being placed in a 50 ml round bottom centrifuge tube at 5°C where it may remain from 6–12 hours, during which a white precipitate settles out. The mixture is centrifuged at 1500 r p m. for 3 minutes, the supernatant is decanted off and the precipitate washed once with cold absolute ether The combined ether extracts are evaporated *in vacuo* leaving a creamy yellow, waxy powder. This is the *crude antithromboplastin*

Purification 200 mg. of this waxy powder is placed in a 40 ml round bottom centrifuge and dissolved in 4–5 ml absolute ethyl ether. To this solution is then added sufficient cold absolute ethanol to achieve maximum precipitation, usually 10–15 ml The ethanol is added slowly with constant swirling of the tube The mixture is placed at 5°C for thirty minutes and then centrifuged at 1500 rpm for two minutes. The supernatant is removed by decantation and the residue dissolved in 4–5 ml absolute ethyl ether Again, cold absolute ethanol is added until maximum precipitation is achieved The above is repeated until a minimum of five precipitations have been accomplished The greater the number of precipitations, the greater the potency of the inhibitor. Following the final precipitation, the residue is dissolved in absolute ethyl ether and the ether solution transferred to a weighed beaker The ether is removed *in vacuo* This material is whiter and flakier than the cruder product

Assay The fractions, as they are obtained from the tissues, are weighed accurately and the residue placed in a small mortar To this is added the correct volume of 0 85 per cent NaCl to make a 1 per cent solution The mixture is rotated with a small pestle until an even suspension (or a complete solution) is accomplished It is then put through a small, hand operated homogenizer seven times The inhibitor potency appears to be directly related to particle size and dispersion, every time it is allowed to go through

4. Antithromboplastin: Preparation and Assay

R. T. CARROLL and L. M. TOCANTINS

By the term *antithromboplastin* is understood an activity present in the blood and extracts of certain tissues, directed against the clot accelerating action of tissue or blood thromboplastin. The exact point of action of the substance or substances responsible for this activity is not entirely clear. As recovered from the tissues by the method to be described, the substance is a lipid; in order to exert its action, a protein co-factor present in the plasma seems necessary. What this co-factor is, is not known It seems that the lipid inhibitor interferes with the formation of blood thromboplastin, as well as antagonizes its action after it is formed.

Two forms of this inhibitor have been recognized: (a) the antithromboplastic lipoprotein of the blood and perhaps of some tissues, and (b) lipid antithromboplastin recovered by extraction of brain, blood and certain tissues with fat solvents This section deals chiefly with the latter type.

Principle: The acetone dried tisue is extracted with absolute methanol, the evaporated residue suspended in 0 85 per cent NaCl, homogenized by exposure to ultrasonic vibration and assayed in an activated plasma clotting system, using a suspension of lipid inhibitor of known potency as a reference standard

Preparation of Tissues for Extraction The organs are obtained fresh, without fixatives, preferably from a body after accidental death. Organs showing gross pathologic changes, or from individuals with chronic debilitating diseases are avoided The organs are freed of vessels, adherent blood clots or other foreign material and rinsed in 0 85 per cent NaCl. In the case of the brain, the meninges and vessels must first be removed completely before the saline rinsing. If the placenta is used, it is perfused with 0 85 per cent NaCl, injecting it directly into the placental artery until a fairly clear solution returns Following the rinsing, if the organs are not to be dried immediately, they are placed in a deep freeze at −10°C. and maintained in the frozen state until ready for the next step, i e, the drying process.

Drying The fresh or thawed out organ is removed from the refrigerator in 30–50 gram portions, placed in a mortar and cut into small pieces To these is then added approximately 150 ml C P or technical grade acetone and the organ is macerated with a pestle, using a constant rotatory, crushing motion When it is felt that the maximum dehydrating effect has been achieved with the acetone sample in use, this is discarded by decantation and fresh acetone is added. The amount of acetone required depends on the

TABLE I· *Effect of Hand-Operated Homogenization and Ultrasonic Vibration on Activity of Lipid Antithromboplastin*

No of Times 1% Suspension of Inhibitor Passed through Hand Homogenizer	No of Minutes Exposed to Ultrasonic Vibration	Activated Clotting Time* (seconds)
0		55
2		68
4		74
5		88
7		92
10		92
	0	58
	1	122
	2	161
	5	191
	10	218
	15	241
	20	246
Control†		15

* 0 1 ml 1 per cent inhibitor suspension, 0 1 ml thrombopl , 0 1 ml citrated plasma, 0 1 ml 0.02 M $CaCl_2$.
† 0 85 per cent NaCl replacing inhibitor suspension

For example, if human lipid antithromboplastin is being tested, human thromboplastin and human plasma should be used in the testing mixture It is also preferable that a dilute thromboplastin be used, if the unknown fraction possesses only slight anticoagulant activity, since a concentrated thromboplastin may well overwhelm a weak inhibitor. Generally speaking, if the original thromboplastin solution is diluted 1–20 with 0 85 per cent NaCl before adding it to the testing mixture, an adequate clot accelerating effect is obtained.

(2) *Plasma.* Blood is collected from a normal donor through a #18 needle into a siliconized syringe containing one hundredth volume of 38 per cent sodium citrate. If the initial venepuncture is not a "clean" one, the blood collected is considered to be worthless and a fresh specimen is collected, preferably from a different vein To insure a good venepuncture, the flow of blood into the syringe should be at the rate of at least 1 cc / second. Following collection, the syringe is tilted back and forth slightly to insure proper mixing with the citrate and then the blood is carefully placed in silicone-coated tubes, removing the needle before allowing the blood to run down the side of the tube. The tube is then placed in a refrigerated centrifuge (temperature maintained at 5°C) and centrifuged at 4500 rpm for one hour The upper ¾ of the plasma is then carefully removed by means of a siliconized dropper pipet into a siliconized tube, care being taken not to disturb the lower layers of plasma The tube is stoppered

the homogenizer its clot delaying activity is enhanced until a maximum effect has been achieved (table 1). As can be seen from table 1, the potency of the inhibitor is increased until a mximum is reached at seven homogenizations, when it levels off. The potency may be further enhanced by exposing the material for a given period at a definite frequency to the Raytheon supersonic vibrator (9 kilocycles 50 watts; type K-223, serial #317). The minimum volume to be exposed should not be less than 5 ml. Below this volume, the results are not reproducible. If the correct volume of a given solution is exposed in the vibrator at a definite frequency for a uniform period of time, duplicate results can be obtained. Different vibrators do not give the same results. As in the case of the hand homogenizer, the longer the period of exposure, the greater the enhancement of potency, until a maximum effect has been achieved (table 1).

For uniformity in handling and comparing with standards, 5 ml of a 1 per cent suspension, exposed to the ultrasonic vibrator (9 kc) for a period of twenty minutes is always used. Following homogenization, the material is maintained at 5°C. until ready for testing. If the material is to be kept over a longer period than 4–6 hours for additional testing, it should be re-homogenized in the ultrasonic vibrator. The standard should, of course, be treated in the same manner Just prior to testing, the suspensions are brought to room temperature and, using 0 1 N NaOH, adjusted to pH 7 2 to 7.4 with a Beckman pH meter Prior to adjustment of pH, the suspension is usually slightly acid (6 5–6 8)

Preparation of reagents for the assay (1) *Tissue thromboplastin.* Three hundred mg of acetone dried human brain powder, prepared as previously described, are extracted for thirty minutes with 5 ml of 0.85 per cent sodium chloride solution, in a 50 ml round bottom glass tube placed in a water bath at 48°C. The tube is rotated for a few seconds at 5 minute intervals The tube is then centrifuged at 1500 rpm for two minutes and the supernatant fluid removed by decantation, centrifuged again in a similar fashion and the somewhat cloudy supernatant decanted off and used A large batch of the thromboplastin is prepared at one time, amounts from 2–3 ml placed in cork-stoppered glass tubes, quick frozen with dry ice, and kept in the deep freeze at −10°C. until ready for use. It is then thawed rapidly by repeated immersion, for a few seconds at a time, under hot running water. Once the thromboplastin has been thawed, it retains its potency for 6–8 hours if placed in the refrigerator at 5°C when not in use When needed, it is brought to room temperature, diluted if desired, then added to the testing mixture. A thromboplastin solution cannot be repeatedly thawed without losing some of its potency. If prepared and kept as described, the thromboplastin solutions maintain a constant potency for months As much as possible, reagents prepared from the same animal species should be used

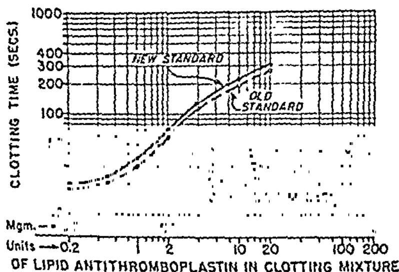


FIG. 1.—Titration curve of a new standard lipid inhibitor on the basis of the curve for the old standard. The curve of activity of the new standard between 40 and 200 seconds is used for interpolating values obtained in testing the unknown samples. Clotting times are converted into units of activity by reading the values in the abscissa corresponding to each time.

Tube No. 1: 0 1 ml. 0.85 per cent NaCl, 0 1 ml. thromboplastin, 0 1 ml. citrated plasma, 0.1 ml. 0 02 M $CaCl_2$

Tube No. 2. 0 1 ml 1 per cent suspension of the new standard lipid inhibitor, 0.1 ml. thromboplastin, 0 1 ml citrated plasma, 0 1 ml 0 02 M $CaCl_2$.

Tubes No. 3, 4, 5, 6, 7 and 8 Same as tube No. 2 except that the standard inhibitor solution is diluted with 0 85 per cent NaCl to obtain, respectively, the following concentrations· 0 5, 0 2, 0 1, 0 05, 0 02, 0 01 per cent The clotting times of the mixtures in tubes 2 to 8 are plotted on the chart (fig 1)

Tube No 9 0 1 ml 1 per cent suspension of *unknown sample*, 0 1 ml thromboplastin, 0 1 ml. citrated plasma, 0 1 ml 0.02 M $CaCl_2$

Let us say that the clotting time of the mixture in tube No. 9 is 52 seconds. This is the activity of 1 mg of the unknown sample To convert this value to units of the standard, the point on the abscissa is located which corresponds to 52 seconds on the new standard curve (Fig 1) This is equivalent to 1 6 units of antithromboplastin activity, or the activity found in 0 08 mg of the standard inhibitor. It may be said then that the unknown sample has 1 6 units of antithromboplastin activity (A U) per mg.

and refrigerated at 5°C. until ready for use on the same day. It is then brought to room temperature and added to the clotting mixture, using a 1.0 ml. siliconized pipet, graduated in 0.01 ml.

Testing. Silicone-coated glassware is used in preparing and measuring the plasma. The clotting mixture [illegible] wing ingredients, always add [illegible]:

[illegible] solution (or 0.85 per cent NaCl)
0.1 ml. human thromboplastin
0.1 ml. normal citrated human plasma
0.1 ml. 0.02 M $CaCl_2$

Calculation: The activity of the unknown is expressed in terms of units per mg., by comparing it with the activity of a lot of lipid antithromboplastin, kept as a reference standard A partially purified lipid antithromboplastin obtained from human brain has been used as the standard In the dry state, at −10°C., the lipid has maintained its original anticoagulant activity for at least two years, when compared, in an activated clotting system, with another anticoagulant (Toronto heparin, 100 units per mg.). One gram of a purified lot of the lipid inhibitor was selected as a standard and granted arbitrarily 20 units of antithromboplastin activity per mg When a lipid extract is to be assayed for antithromboplastin activity, 50 mg. of the standard is mixed with 5 ml. of 0.85 per cent NaCl and, after hand homogenization, exposed to the water-cooled ultrasonic vibrator for 20 minutes. The unknown sample is prepared in the same manner. A concentration/activity curve for the standard antithromboplastin is worked out each time, utilizing as a clotting substrate the same plasma and thromboplastin employed in assaying the antithromboplastin activity of the unknown extract In figure 1 the line is plotted which expresses the relationship between activity (clotting time) and amount of antithromboplastin of a partially purified material from dried human brain

As can be seen by a study of the curve, the concentration/[illegible]y ratio, plotted on a logarithmic scale, is [illegible] poses of interpol[illegible] for activi[illegible] [illegible] 40 and 200 seconds on the ch[illegible]. [illegible] unknown sample has an initial clotting time above (or below) these limits, it is diluted (or stronger solutions are used) so that the values fall into the useful section of the curve

Example for calculating the units of antithromboplastin activity of an unknown sample: The reagents (thromboplastin, plasma, lipid inhibitor and 0.02 M $CaCl_2$ solution) are prepared as described Silicone-coated glassware is used throughout Measurement of the clotting time is done in duplicate at 38°C

5. Heparin: Methods of Assay

L. B. JAQUES

Principles Heparin may be detected by direct or indirect methods. Indirect methods are (1) clotting time determinations and (2) protamine (toluidine blue) titration. Direct methods involve the extraction of the heparin by (1) the Charles and Scott procedure for tissues or (2) the Monkhouse and Jaques procedure for blood, followed by identification and measurement of the heparin obtained Two problems are involved in such measurements—identification and quantitative determination. These are apt to be confused. The only satisfactory means of identifying heparin is isolation, followed by observation of a number of properties. crystalline form, solubility, sulphur content, optical rotation, specific biologic activity, specific metachromatic activity, etc No one property is unique to heparin but a combination of these measurements gives a dependable result It should be appreciated that impurities in the extract can seriously interfere with measurements, either directly as when the presence of thromboplastic activity causes a low reading in a clotting test or indirectly as when the presence of amino acids interfere with metachromatic readings All tests must be conducted by direct comparisons with standard beef heparin. Since different species show different values for the ratio of anticoagulant activity (clotting time effect, etc) metachromatic activity (protamine titre, protein combining power), this ratio is very useful for identification and also for evaluation of the reliability of the data obtained

Use of Clotting Times[1] Where heparin is being injected and it is known that other changes will not occur in the clotting system, this is the simplest procedure The technic is discussed on page 29 It is possible, by drawing blood in silicone coated syringes and glassware and mixing with known quantities of heparin, to construct a standard curve for converting clotting times to heparin concentrations

Protamine Titration Protamine is a basic protein Heparin is acidic and forms a salt with protamine Both are anticoagulants However, on adding varying quantities of protamine to heparinized blood, an amount of protamine can be found which restores the clotting time to normal (other complex bases, such as toluidine blue and Polybrene, can be used in place of protamine)

Reagents and Equipment Eight mm test-tubes, rack, water-bath, stop watch, 5 ml siliconed syringe with Arquad-treated needle Protamine solutions, 1 mg /ml and 0 1 mg /ml in isotonic saline, with pH adjusted to 7 0

In order to assay the *total* units of antithromboplastin activity in a given sample of blood, plasma or tissue, the yield of the extract in mgms. is multiplied by the units of activity per mg. of the extract.

Before the supply of standard inhibitor is exhausted, a new lot should be prepared and standardized in terms of the old one. A relatively large amount of lipid inhibitor should be prepared, in order to eliminate the necessity for frequent restandardizations To obtain a large yield, 30 grams of dried brain powder is used as a start and to it are added 1200 ml. of absolute methanol. Following the filtration, distillation, removal with absolute ethyl ether and the separation of the ether insoluble material in the refrigerator, the final yield should be 2.0 to 2.5 grams A 1 per cent suspension is prepared in 0 85 per cent NaCl, homogenized and an activity curve is worked out as previously described At the same time, using the same thromboplastin and plasma as basic reagents, an activity curve is worked out for the old standard lipid inhibitor. The two curves (fig. 1) are compared and a new unitage is granted to the new lot in terms of the units of the old standard.

Precautions and Sources of Error: (a) Use of technical grade methanol instead of *C. P Absolute Methanol* in the extracting procedure. (b) Extraction at room temperature, if extraction is allowed to proceed at temperatures higher than 10°C, coagulant materials are extracted and the anticoagulant potency of the assayed materials is lower. (c) Imperfect homogenization of the suspension If the lipid suspension is not rendered almost water clear by this process, the ultrasonic homogenizer is probably defective (d) Allowing material to stand too long (over 2 hours) after homogenization, before it is tested (e) Use of heterologous thromboplastins or plasma substrates (f) Use of unstable, hypercoagulable plasmas as substrates

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used recalcified citrated human blood. Astrup and Galsmar (1944) who used ox plasma, found that with this system, synthetic sulfonated polysaccharides showed much greater activity than heparin, although their anticoagulant activity was much less, so that some of these tests do not measure anticoagulant activity at all Jalling et al.[3] discuss these methods and report discrepancies observed with their use. The U S. Pharmacopeia has adopted the sheep plasma method of Foster as the official test. The British Pharmacopeia gives the cat blood, sheep plasma, and other methods as alternatives

With all methods which use a clotting system for the assay of heparin a choice has to be made between estimating degree of clotting with varying amounts of heparin at a fixed time and estimating the clotting times with varying amounts of heparin The factors involved in this choice are discussed by Jaques and Charles [1]

It must be appreciated, for physiologic and pathologic studies, that many of these methods do not distinguish between heparin and its metabolized or inactive forms, and that many of them also are of doubtful specificity for heparin. On both these points, the original assay procedures using fresh whole blood, while not free from criticism, are more reliable than the other methods

Heparin Units Howell originally defined the activity of heparin in terms of units, one unit being that amount which prevented the clotting of 1 ml of cat's blood in the cold for 24 hours A number of factors such as temperature are not made clear in this definition and can cause as much as a five fold difference in activity However, the unit used by Charles and Scott is as close to the original unit of Howell as can be determined at the present time. The international unit which was adopted in 1942 by the League of Nations is of the same order of potency as the Charles and Scott unit and, therefore, of the original Howell unit [8] It is the activity of $\frac{1}{130}$ mg of the international standard powder which is prepared in the form of the sodium salt of heparin

All assays must be conducted against a standard preparation of beef heparin assayed carefully by the same procedure against a sample of the international standard heparin powder In the U S A this can be obtained from the U. S P Reference Standards, 46 Park Avenue, New York 16, N Y and in other countries from the similar authority

Since activity varies with species, it is advisable in physiologic and pathologic studies to express heparin concentrations, where possible, in milligrams This requires the isolation of heparin from the species under study in order to establish its absolute unitage in relation to the beef heparin standard

Procedure: Eight mm. test-tubes are cleaned by washing in soap and water, rinsing thoroughly with distilled water, dried and finally cleaned in chromate-concentrated sulphuric acid solution. After rinsing and boiling in distilled water, they are rinsed and dried. The test-tubes are placed in a rack in a water-bath, at 37°C. in groups of six. The following amounts of protamine are measured into the tubes, 0 5, 0.1, 0.05, 0 02, 0.005, 0 0 mg. Volume is made up to 0 5 ml. with isotonic saline About 4 cc. of blood is taken by vein puncture. The needle is removed, the half ml. discarded and 0.5 ml of blood added to each tube, mixed and clotting times measured. In addition to reading the 1st tube clotted, the second and third are noted, as this allows estimation of an end-point occurring between tubes. For toluidine blue, the test is performed similarly but substituting the dye for the protamine.

Sources or Error: Each lot of protamine must be standardized, as the ratio changes with the lot number Protamine has other effects on the clotting system. In addition to its inhibitory action and power to neutralize heparin, it clumps and destroys platelets. It is therefore not specific. Also, the quantity of protamine is the quantity required to depress heparin activity in blood, not the amount to combine with heparin. In using the test, a safe rule is that a clotting time three times the normal value or more if reduced to normal by adding protamine can be attributed to heparin and the titration reported in heparin units Lesser changes should be reported as questionable unless supported by other tests.

Biologic Assay Procedures for Heparin[1] As with any therapeutic substance in use, the anticoagulant activity of heparin must refer to its activity on the actual biological system itself, i.e., the clotting of whole blood. This was the principle adopted by Howell in his original cat assay and also, with modifications, by Charles and Scott. Jorpes used beef blood, and Schutz, rabbit blood It can be extended for special problems, to human blood It would be advisable to limit the term "anticoagulant activity" to such tests on the clotting of fresh whole blood

Because of inability to obtain whole blood in many laboratories, it is usual now for many purposes to measure some other property of heparin. Fischer and Schmitz in 1932 used inhibition of clotting of chicken plasma, since it is relatively simple to collect chicken blood without any anticoagulant and after separating off the cells, preserve the plasma for the assay. This was also used by Dam and Glavind in 1939 Jaques and Charles used the clotting of oxalated blood by thrombin Many have used plasma clotted with calcium and thromboplastin Reinert and Winterstein (1939) and Foster (1942) used ox plasma; Kizenga, Nelson and Cartland (1943) used sheep plasma, MacIntosh (1941) used horse plasma and excess thromboplastin. P. Astrup (1947) used citrated human plasma, McGoon (1950)

standard as the respective dilutions of the two. Where the readings of the standard lie between those intermediate dilutions, it is possible to estimate the potency of the unknown but where they lie beyond those of any of the dilutions of the unknown it is necessary to make further assays using new dilutions of the unknown.

The Thrombin Method of Assay for Heparin

Reagents: Citrated beef or dog blood. Thrombin: 1 ampoule (5000 units) of "Topical Thrombin" dissolved in one-half volume of 0.85 per cent NaCl and made up to volume with 50 per cent glycerol. This is kept in refrigerator and diluted with 0.85 per cent NaCl as needed. 8 mm. wide (i.d.) test-tubes. Water bath at 25°C. Standard heparin: 0.1 units per ml. prepared from reference standard.

Procedure: The thrombin solution is standardized by adding varying amounts to a clotting system composed of 0.5 ml. of blood, 0.2 and 0.3 ml. of standard heparin and 0.85 per cent NaCl solution to give a total volume of 1.0 ml., and examining the tubes after standing fifteen minutes at 25°C. That amount of thrombin which is just sufficient to cause clotting with 0.2 ml. of heparin and not with 0.3 ml. is then taken for the assay. The stock thrombin solution is diluted as required to contain this amount of thrombin in 0.1 ml. For the assay, 0.20, 0.225, 0.25, 0.275 and 0.30 ml. of the heparin standard are taken in carefully selected tubes (as described for the Howell method). The volume is made up to 0.4 ml. with 0.85 per cent NaCl and 0.50 ml. of oxalated beef blood is added. In another series of tubes, equivalent amounts of the unknown, as found by preliminary assay, are taken. The tubes are allowed to stand in a water-bath at 25°C. for exactly ten minutes, to come to temperature equilibrium, and then 0.1 ml. of the thrombin solution is added to each tube, the system being mixed immediately by inverting the tube twice. The tubes are allowed to stand in a constant temperature water bath at 25°C. for fifteen minutes and then read. Below a certain concentration of heparin, the clot is found to be solid and slides down the tubes. Above this concentration, which is the end point, the clot breaks up and with higher concentrations, of course, no clot is formed. The end point will be found between one pair of tubes. This must occur between the second and third or third and fourth tube. If not, it is necessary to make further assays using new dilutions.

Fischer Assay for Heparin

Reagents: Chicken blood is collected by paraffined syringe from a wing vein or by paraffined cannula from the carotid artery, chilled, centrifuged in paraffined vessels, and the plasma diluted with an equal volume of

Howell Method (as Modified by Charles and Scott) of Assay for Heparin

Reagents· Carefully selected 8 mm. Widal tubes marked at a volume of one ml. are used. The tubes are grouped according to the bore and only those of one group are used in one set of assays A standard heparin solution containing 2.5 International units per ml. is used All solutions are made up in 0.85 per cent NaCl solution containing 0.3 per cent of tricresol. A cat is anesthetized by intraperitoneal injection of amytal (80 mg sodium amytal/Kg.). One carotid artery is exposed and a clean glass cannula inserted.

Procedure: The unknown solution is diluted to approximately the same strength as the standard, as indicated by a preliminary assay. Four dilutions of the unknown in this range are taken, such that there is a difference of 10 per cent between each dilution 0.1 ml, 0.2 ml, 0.3 ml. of each solution (the standard and four dilutions of the unknown) are measured into each group of three tubes, and the volume is adjusted to 0 3 ml. with 0.85 per cent NaCl solution. These are placed in a rack holding 30 tubes The tubes are filled to the 1 ml. mark with blood from the cannula. All the tubes in the rack containing only 0 1 ml. of the solutions are filled first, then those with 0 2 ml, and then those with 0 3 ml. The standard solution is taken first, then strongest dilutions of unknown followed by other dilutions in order. As each tube is filled, it is mixed by inverting twice in such a manner that the blood covers the inside area of the tube. Three racks may be filled at the same time, but each set must have its own standard and be filled separately The racks are placed in a water bath at 25°C for two hours, and the tubes are covered to prevent evaporation Since with an occasional cat, all the tubes will be clotted after two hours, it is advisable to inspect the tubes after an hour and a half, and if all the tubes of the weakest dilution appear to be almost completely clotted, to read the tubes then; such a condition is readily seen, since settling of the red cells with the resultant buffy coat can normally be observed whereas, if the blood is clotting too rapidly, this does not occur The tubes are read by tipping each tube and judging the degree of clotting A complete clot which does not break upon tipping is recorded as +, whereas completely fluid blood is recorded as – Intermediate stages representing decreasing degrees of coagulation may be designated by D +, D, dd, d At least four intermediate stages can be distinguished, and with practice, eight can be detected After completing the reading, the data is examined, to compare the standard with the unknown In a good assay the readings for the tubes containing the standard must match with those for one intermediate dilution of the unknown The ratio of the potency of the unknown is to that of the

U.S.P. Assay

Reagents: Standard solution of heparin. Weigh accurately about 50 mg. of U.S.P. Heparin Sodium Reference Standard previously dried at 60°C. over phosphorous pentoxide to constant weight, and dissolve in sufficient 0.85 per cent NaCl solution to yield a concentration of 1 mg./ml. Store in a tightly-stoppered vessel in a cool place and use it for no longer than 3 months after preparation.

Preparation of plasma. Collect blood from sheep directly into a vessel containing 8 per cent sodium citrate solution in the proportion of one volume to each 19 volumes of blood to be collected. Mix immediately by gentle agitation and inversion of the vessel Promptly centrifuge the blood and pool the separated plasma To a 1 ml. portion of the pooled plasma in a clean test tube, add 0.2 ml. 1 per cent calcium chloride solution and mix. Consider the plasma suitable for use if a solid clot forms within 5 minutes. To store plasma for future use, subdivide the pooled lot into portions not exceeding 100 ml. in volume; freeze the portions at −20°C. or below and store at a temperature not exceeding −8°. For use in the assay, thaw the frozen plasma in a water bath not exceeding 37°C Remove particulate matter by straining the thawed plasma through a coarse filter

Procedure: Weigh accurately about 25 mg. of heparin sodium, previously dried at 60°C. over phosphorous pentoxide to constant weight, in sufficient 0 85 per cent sodium chloride solution to give a concentration of 1 mg. per ml

Determine by preliminary trial, if necessary, approximately the minimum quantity of *standard solution of heparin* which when added in 0.8 ml. of 0 85 per cent NaCl solution, maintains fluidity in 1 ml. of prepared plasma for one hour after the addition of 0 2 ml. of one per cent calcium chloride solution This quantity is usually between 01 and .015 ml of the Standard solution On the day of the assay, prepare a test dilution of the Standard solution such that it contains in each 0.8 ml. of 0 85 per cent NaCl solution, the above-determined quantity of the Standard solution

In the same manner dilute the solution of heparin sodium prepared as directed above.

Clean hard-glass 13 × 100 mm. test tubes by immersion overnight in chromic acid cleansing mixture (page 628, U S P. XIII) Fit the tubes with paraffin-coated corks To a series of these tubes add graded amounts of the test dilution of the Standard, selecting the amounts so that none exceeds 0 8 ml and so that they correspond roughly to a geometric series, in which each step is approximately 5 per cent greater than the next lower. To each tube so prepared, add 1 0 ml of prepared plasma and sufficient 0 85 per cent NaCl solution to make the total volume 1 8 ml. Add 0 2 ml.

Ringer's solution. The thromboplastin is prepared from chicken breast muscle, following the specifications of Schonheyder.

Procedure: In the assay 0.25 ml. of diluted plasma +0.05 ml. of heparin solution + 0.05 ml. of thromboplastin are mixed in a 8 mm. test tube and placed in the Fischer test tube rack in a water bath at 37°C. The tube is examined by tilting at 10-second intervals. A dilution of thromboplastin is taken, such that a clotting time of 30 to 90 sec. is obtained with the plasma plus Ringer's solution. For the assay, a series of tubes containing various dilutions of unknown and standard heparin in Ringer's solution are prepared, including a tube without heparin, and the clotting times determined. It is not clear from Fischer's description whether he used a set of tubes of the standard heparin solution each time or not. This is a first essential for the assay, since Astrup and Astrup found that the heparin activity of a solution as measured by the value of K (see further on) varied considerably with different plasmas, and our data indicate that it changes during a day's assays Hence, it is necessary always to compare the potency of the unknown with that of the standard set up at the same time. Four dilutions of the standard are made up, such as to give measurable clotting times, and also four dilutions of the unknown of approximately the same potency as the dilutions of the standard as determined by preliminary assay, 0.05 ml of each of these is added to the plasma in eight clotting tubes and to a ninth tube, Ringer's solution is added To avoid any bias due to the order of mixing, the order of the tubes is changed in each assay, before adding the thromboplastin In order to initiate clotting simultaneously in all the tubes, the empty tubes are placed in a bath of ice and water. The plasma, heparin and thromboplastin are added in this order and mixed while still in the ice bath. The rack is then transferred to the bath at 37°C and the clotting time reckoned from the time of immersion in the 37°C bath This procedure gives consistent results and shows a linear relation between heparin concentration and log of clotting time as required by Fischer and Astrup

Fischer reports the activity of heparin by its K value, the slope of the line obtained by plotting log clotting time against heparin concentration K is found by the method of least squares, the clotting time with zero heparin being included The heparin activity of the unknown is then calculated from the ratio K (unknown). K (standard) Typical values of K_u and K_s are· 3 95 and 3 97, 4 24 and 4 34, 4 39 and 4 61 These were three assays of a standard solution conducted on the same plasma in the course of eight hours. It can be seen that the K value changes as the plasma ages The potency of the unknown in the three assays was 99 5, 97 7, 95 2 (theoretic —100 0)

activity can only be applied if the shift in absorption spectrum is measured, and if the pH of the reaction is below 10. The dyes, Azure A and toluidine blue have been used as (1) precipitating agents for heparin (2) antidotes for heparin (3) metachromatic agents. Their use as (1) has been discussed elsewhere; (2) is the same as the protamine titration for heparin (see page 383) Azure A and toluidine blue have an absorption maximum at 620 mμ. On the addition of heparin, this is depressed and a new band appears at 500 mμ. The change in these bands is a function of the heparin concentration but at 550 mμ, the presence of heparin does not affect absorption. These dyes are also acid-base indicators above pH 10, giving a red color with alkali Hence, measurements in alkaline solutions are not measurements of metachromasia. As examples of these methods, the turbidity method of Winterstein and the metachromatic method of Jaques et al are described As already indicated, these methods do not readily distinguish between heparin, its precursors and metabolic products, and require checking by other methods.

Turbidity Method of Marbet and Winterstein

Reagents. Four per cent solution of 2-Dimethylamino-methyl-dibenzofuran-hydrochloride (Roche) Ten per cent hydrochloric acid Kieselgur, in case of urine containing protein Nephelometer

Procedure. Four ml sample is placed in nephelometer tube, one drop of 10 per cent HCl added and reagent added to the 8 ml. mark. The contents are mixed, the tube allowed to stand 5 minutes and the turbidity read in a nephelometer. Marbet and Winterstein describe a paper nephelometer graduated in mg per cent heparin obtainable from F. G Hoffmann-La Roche & Company A G., Basel While developed for urine, the method can be used for other protein-free heparin solutions

Metachromatic Assay of Heparin

Reagents: 100 mg per cent Azure A (certified biologic stain) Diluted before use to 10 mg (actual dye) per cent. Borate buffer- 0 10 M H_3BO_3-0 15 M NaCl-NaOH, pH 8 5

Procedure The unknown heparin solution is measured into the colorimeter cell, and the volume made up to 2 0 ml with the NaCl solution. Two ml of buffer is added and 1 0 ml of the Azure A solution, mixed and the light absorption measured at 500 mμ. The heparin concentration is determined from a standard curve constructed with known quantities of heparin Repeat in triplicate or more for two or more concentrations on the steep part of the curve

The selection of a suitable colorimeter is important The Lovibond tintometer has proved satisfactory With this, the complementary color of

of 1 per cent calcium chloride solution; note the time, immediately stopper each tube and mix the contents by inverting three times in such a way that the entire inner surface of the tube is wet

In the same manner use the test dilution of the preparation under assay in a series, completing the entire process of preparing and mixing the tubes of the Standard and of the preparation under assay, respectively, within 20 minutes. Exactly one hour after the addition of the calcium chloride, determine the extent of clotting in each tube, recognizing three grades (0.25, 0.50 and 0.75) between zero and full clotting (1.0) If no tube of a series is graded more than 0.5 or if no tube is graded less than 0 5, repeat the assay using appropriately modified test dilutions

Calculation of the Potency Note, or ascertain by interpolation, the respective volumes of the test dilutions of the Standard and of the preparation under assay required for the 0.5 grade of clotting From these volumes determine the weight, in mg., of U S P. Heparin Sodium Reference Standard to which 1 mg. of the heparin sodium is equivalent in activity

Chemical Assays[1]

The multiplicity of reactions given by heparin with complex bases and proteins have made possible the development of a great number of assay procedures based on physical and chemical tests Fischer and Schmitz devised an assay based on the effect of heparin on turbidity of casein. This still appears to be as satisfactory an assay as any developed later Various alternatives of this procedure have been developed MacIntosh for example, used the amount of toluidine blue remaining in solution after precipitation by heparin. This has also been used by Copley and Whitney (1944) and Trethewie and Melvin (1945), while Gibson has measured the amount of dye precipitated by heparin Winterstein has recently developed a dibenzofuran derivative (No. 1261) which gives stable suspensions with heparin and hence turbidity measurements can be used.

While the metachromatic properties of heparin are rather unique, the only quantitative method using it as a basis for assay is that described by Jaques, Mitford and MacDonald, and Jaques, Monkhouse and Stewart[1] Copley described a crude semiquantitative method using this principle. As a qualitative test for identification of heparin, it has been used very successfully by Scandinavian workers in many investigations

Metachromasia refers to a shift in the absorption spectrum of a dye in the presence of certain complexes. Heparin and related acid polysaccharides show this property to a high degree and in the presence of sufficient sodium chloride which depresses the activity of hyaluronic acid and similar substances, the method is fairly specific for the heparin series of compounds. It should be emphasized that the term metachromatic

Procedure: The tissue is minced and allowed to stand for autolysis at 25°C. for 24 hours. The sodium hydroxide and saturated ammonium sulphate solutions are then added and the mixture heated to 50°C. in a water bath, kept at this temperature for 30 minutes, then heated to 70°C. The tissue is then filtered immediately through Chain cloth and allowed to drain. Large quantities of tissue are strained through cheese cloth or open wire mesh The filtrate is acidified to pH 2 0 to 2.5 with sulphuric acid, heated to 65°C. and centrifuged. The precipitate is washed with hot water containing sufficient sulphuric acid to pH 2 0.

The precipitate is then extracted at room temperature for 20 hours with 95 per cent ethyl alcohol. The mixture is then centrifuged and the alcohol removed. The precipitate is dissolved in 15 ml of water, sodium hydroxide added to pH 8.4, trypsin added and the mixture incubated at 37°C. for 36 hours with continuous stirring. The pH is adjusted to 8.4 by adding dilute NaOH. For the first hour, the pH must be checked every few minutes with a Beckman glass electrode Preservative is then added and the digestion continued for 36 hours. The mixture is acidified (HCl to pH 6 0) and two volumes of 95 per cent ethanol added The precipitate is removed by centrifuging and then extracted by repeated washing with hot acetone until all fat is removed This gives a crude heparin powder which can be subjected to identity tests (chromatography, etc.). However, unless the tissue has a high heparin content, it is necessary to carry out further purification procedures For quantities of tissue greater than 500 Gm it is necessary to substitute filtration for centrifugation

For identification studies in most tissues it is necessary to process 50 to 75 pounds of tissue When this is done the extractions are carried out in a double jacketed steam kettle (Dopp) Filtration through cotton cloth is used at each stage The heating at various stages of filtration and centrifugation keep the fat fluid, so that it will be discarded in the filtrate

Purification depends upon the particular tissue and species being investigated Purification procedures commonly used are (1) treatment with ammonium carbonate (2) charcoal (3) Lloyd's reagent (4) benzidine (5) precipitation as brucine salt and (6) precipitation as the barium salt

Ammonium Carbonate The crude material is dissolved in alkaline water at an approximate concentration of 6 per cent, 10 per cent ammonium carbonate added, heated to 70°C and either centrifuged or filtered through celite on cloth on a Buchner funnel Before further stages, the carbonate is removed by slowly adding acetic acid, care being taken not to lose solution by frothing. This is one of the most useful methods for treating heparin

Charcoal Charcoal is useful for removing coloring matter Charcoal approximately equal to the original weight of crude is added, the mixture

the absorption band is matched visually against standard glasses to give a curve: Lovibond red units vs. heparin. Photoelectric colorimeters must have a high dispersion, allowing the use of a small spectral band width The Coleman Jr. Spectrophotometer with absorption at 510 mμ may be used for rough quantitative work. For accurate work, a Beckman DK2 spectrophotometer with temperature control of the cell compartment is required. The dye solution is made up to 5 mg. per cent and the difference in absorption of dye and dye plus heparin at 500 mμ determined.

Sources of Error: These may be due to the dye, the sample or the measuring instrument. While the reaction was first described for toluidine blue, authentic samples of this dye do not give the reaction. The reaction is due to Azure A. However, dye samples do change with time. The stock solution must be kept in the cold and renewed monthly or even earlier, whenever 80 per cent of the stock has been used up. Only certified dye can be used Even this material on chromatography is found to contain two non-metachromatic components. It is advisable to determine the optimal dilution for each lot of dye. Many impurities in crude heparin preparations interfere with the reaction. If a photoelectric colorimeter is used, the absorption spectra for dye, and dye + heparin must be run with the instrument, to determine the position of the absorption band in the particular instrument and whether the instrument will detect it with sufficient sensitivity. Finally, it should be remembered that the absorption band can be made to disappear by heat and by alcohol, and that the pH of the mixture should be checked when dealing with many solutions.

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[1]Jaques, L B, and Bell, H J. Determination of heparin Methods Biochem Anal *7*: 253, 1959

6. Heparin: Preparation and Purification

L. B. JAQUES and H. J. BELL

The following is the method devised by Charles and Scott[1] for extraction of heparin from the tissues (for other methods, see Jaques and Bell[1]).

Reagents: For each 100 Gm. of tissue: N/2 sodium hydroxide (150 ml.), saturated ammonium sulphate (18 ml.), 95 per cent ethanol, Difco trypsin (200 mg.).

ammonium salt. The heparin is dissolved one gram per 5 ml of alkaline water, 1.5 ml. of 20 per cent ammonium carbonate solution is added, the solution heated to 65°C. and then centrifuged. The precipitate is washed with a 6 per cent solution of ammonium carbonate solution at 65°C., the wash added to the original supernatant and the whole acidified with acetic acid, care being taken at this stage not to lose solution due to rapid evolution of CO_2. The volume is then made up to 12.5 ml and samples are removed for crystallization One to two ml. samples are diluted to 5 ml, 1.4 ml of 10 per cent barium acetate solution added and the tube is heated to 65°C. The precipitate at this stage is barium sulfate and is removed by centrifuging The clear solution is then warmed to 65°C. and 1.2 ml. of glacial acetic acid added. This is then allowed to cool slowly to room temperature. A heavy precipitate comes out, starting at the bottom and the center of the tube at about 40 to 45°C. Under the microscope it appears crystalline in forms of rosettes and sheaves. Depending upon the purity of the sample, the impurities present and the species of heparin, the condition of crystallization may require adjustment. The chief significant factors in this regard are concentration of barium, acetic and heparin. When other trials are required the concentrations of barium and acetic should be varied in steps of about 10 per cent. With different species of heparin the final concentration of heparin required varies from 1–6 per cent. For preservation of heparin samples for future reference it is advisable to convert them to the neutral sodium salt. To do this, the barium is removed with ammonium carbonate as described for preparing for crystallization, the carbonate removed by adding acetic acid, the solution then carefully adjusted to pH 7.0–7.1 with NaOH, and the heparin precipitated by pouring the solution into three volumes of 95 per cent methyl alcohol The resulting precipitate is centrifuged, washed twice with ethanol, washed twice with ether and dried This form of heparin is quite stable. Heparin is hygroscopic and should be stored in tightly sealed containers. Dried over calcium chloride at 25°C., it contains 12–15 per cent of water of crystallization In comparing samples of heparin for specific activity, allowance must be made for water and base content of each sample Thus, water and barium make up approximately 35 per cent by weight of the barium salt

Paper Chromatography of Heparin: This can be used as an identity test for heparin; to demonstrate the presence of certain impurities in heparin; to remove some impurities from heparin. The paper chromatogram may also be used to assess methods for the isolation and purification of heparin and to scan tissues for heparin

Apparatus and Reagents Required Micropipette, Pyrex crystallizing dish, Pyrex chromatogram chamber, "paper for chromatography," 1 per

heated to 70°C. and after contact from 30 minutes to five hours is filtered with celite. Time of contact must be carefully controlled by trying small samples since heparin can be absorbed by charcoal. There is great variation in different samples of charcoal in this regard British Drug House charcoal is satisfactory but each batch must be standardized The charcoal treatment may be repeated adding 1 per cent acetic acid before each treatment

Lloyd's Reagent: Originally used by Howell for heparin, is one of the most useful materials, since it rarely absorbs heparin. Best results are obtained if the solution is on the acid side and left in contact with the Lloyd's in the cold overnight Five per cent Lloyd's reagent is added and removed either by centrifugation or filtration through linen-paper pulp-paper. This may be repeated several times adding one per cent acetic acid between each treatment.

Benzidine· This is used to precipitate heparin, but some purification of crude heparin must be achieved before applying benzidine. It is best to apply this process to material which is running 20 to 40 per cent heparin The solution is acidified (clear) and five per cent benzidine HCl is added in fractions until no further precipitate is obtained with the benzidine. The precipitate is centrifuged, supernatant discarded and the precipitate washed with a diluted solution of benzidine. The precipitate is hydrolysed by adding water and concentrated ammonia and heating to 70°C. During the heating the mixture is checked frequently with litmus. The litmus should turn blue and then the spot slowly turn red on standing, due to hydrolysis of the benzidine salt The solution must be kept strongly alkaline. When hydrolysis is completed, it is chilled to 30°C. and the benzidine removed by centrifuging and filtering it through line-pulp-linen Some trace of benzidine remains in the heparin solution and this can be removed by absorption on Lloyd's reagent, which oxidizes the benzidine to an insoluble blue compound

Brucine: To precipitate the brucine salt of heparin, a half volume of 5 per cent brucine phosphate, pH 5 5 is added to the heparin solution at pH 5.5. The precipitate is collected and can be washed and dried with absolute alcohol and ether or hydrolysed directly It is hydrolysed in 0 05N NaOH at 70°C. for 15 minutes (see Jaques, Monkhouse and Stewart)[1]

Barium· The barium salt of heparin in concentrated solution is soluble warm and insoluble cold This can be made use of for precipitating heparin when the heparin concentration reaches 20 to 40 per cent Once purification to the stage of 40 per cent heparin is reached, then crystallization is the method of choice This gives the highest degree of purification in a single step of any of the methods For crystallization the heparin must be treated with ammonium carbonate to remove calcium and barium and to form the

on elution. In interpreting paper chromatograms of heparin, it should be remembered that salts often separate into ions, and interactions occur between substances and impurities in the solvent or in the paper.

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[1] Jaques, L. B., and Bell, H. J.: Determination of Heparin Methods Biochem Anal 7: 253, 1959

7. Microelectrophoresis of Heparin[1]

L. B. JAQUES, R. E. BALLIEUX and C. von ARKEL

Object: To identify and measure heparin.

Principle. Van Arkel, Ballieux and Jordan[2] have reported the adaptation to mucopolysaccharides of microelectrophoresis. As agar is metachromatic, it is necessary to use agarose (the sulphate-free component of agar). With this, heparin can be seen as a distinct metachromatic band and can be distinguished from other mucopolysaccharides by its higher rate of migration and more pronounced metachromatic colour. 0 01—0 02μg. of heparin are demonstrable. Combined staining for proteins and mucopolysaccharides shows complete separation of heparin and proteins (e g, human serum proteins) by the microelectrophoresis The electrophoresis is conducted at pH 8 6 and 11°C.

Apparatus, Reagents and Sample Preparation· Electrophoresis is conducted on microscope slides using the apparatus of Wieme.[3] A commercial form of the equipment is obtainable from Meyvis & Co , Bergen op Zoom, Holland Optical density of the red-purple spots can be read in a Chromoscan densitometer (Joyce, Loebl & Co Ltd , Newcastle-on-Tyne, England), using an Ilford 654 filter

Agarose is prepared from Difco Noble agar. This is done by acetylating, fractionating with organic solvents, and deacetylation (method of Araki[4]). Alternatively, the agar is precipitated with cetyl pyridinium chloride (Hjerten[5]) and centrifuged or filtered at 65°C Agarose is dissolved in barbiturate buffer (10 4 Gm sodium barbiturate and 1 84 Gm barbituric acid in 1000 ml. distilled water, pH 8 6). The agarose solution is poured on slides to give a thickness of 1 mm Slides should be prepared the day before using One per cent Cetavlon* is prepared in distilled water and diluted 1:10 before use Forty mg toluidine blue is dissolved in 20 ml water and 80 ml of dry acetone. Three hundred mg lissamine green is

* Cetyltrimethylammonium bromide (hexadecyltrimethyl ammonium bromide, EKT 5650)

cent glucose solution, aqueous ethanol (usually 45–60 per cent) containing 1 per cent ammonia (by volume).

Steps in Performance of Test: Wash an 18 × 22 inch sheet of Whatman No. 1 or No. 2 "paper for chromatography" with water of high purity, and dry; reflux with EtOH and then with water. Mark a starting line 5 inches from the end of the sheet with a glass rod stylus and line the paper vertically Apply with a micropipette to the paper Heparin (0 25 mg. per spot) and one glucose standard spot (100 μg per spot), the volume not exceeding 2 5 μl. at one time. Place the outer spots 1½ inch from the sides of the paper and all spots far enough apart that no overlapping after chromatography occurs. An area is left free of spots to provide blanks for analysis of the paper. After the spots dry, the paper is folded into a cylinder held with two glass pins and put in a crystallizing dish containing 70 ml of the water-EtOH-ammonia solvent system Put the crystallizing dish on a glass plate upon which are placed several small vessels containing water and the solvent system Then cover with a pyrex chromatogram chamber, previously supersaturated in respect to water and the water-EtOH-ammonia solvent system. The chamber is placed away from drafts. When the solvent front reaches ½ inch from the edge of the sheet, mark the solvent front, remove the chromatogram and dry at room temperature. Cut the test section into two strips, one strip containing a heparin spot and the other the glucose standard, stain the heparin spot with Azure A in EtOH (40–50 mg per cent) and the glucose with benzidine Under controlled conditions, the distance the unknown travels divided by the distance the reference glucose travels gives a constant value, the R_G value If the test strip indicates little or no trailing has occurred, the paper is further divided into strips, each strip containing a heparin spot and treated with reagents—alizarin ammonia, 8-hydroxyquinoline and kojic acid, ammoniacal silver nitrate and fluorescein, mucicarmine, etc. to locate the substances on the paper For quantitative chromatography, elute the papers with water of high quality: 70 ml. for a strip 4 × 8 cm Pool the eluates from different sheets for a given R_G fraction, reduce to small volume and dry to constant weight. The residues are then used for assay and chemical analysis. Results obtained must be corrected for values obtained with the suitable blank When only qualitative chromatography is required, the paper need not be treated before use.

Sources of Error: The chief disadvantage of paper chromatography is the considerable impurity obtained from the paper on elution and hence the high blank value of the eluate with many methods of analysis Another difficulty encountered is that the presence of heparin on the chromatogram would appear to increase the amount of impurity obtained from the paper

SECTION B

HEMORRHAGE

METHODS FOR THE EVALUATION OF NORMAL AND ABNORMAL HEMOSTASIS IN VIVO

dissolved in 100 ml. 1 per cent acetic acid. One per cent acetic acid is used for washing.

Heparin solutions should contain heparin equivalent to approximately 1 mg. commercial heparin/ml. Tissue extracts may require preliminary deproteinization, although suspensions may be applied to the slide. Urine requires fractionation and concentration by the benzidine procedure of Astrup[6] and plasma by the phenol procedure of Monkhouse and Jaques (*see above*). The larger amounts of chondroitin sulphuric acid present may blur the heparin spot. This can be removed by precipitation of the heparin with 9 volumes of acetic acid. A reference heparin solution is required This should be a single bottle of a good commercial heparin kept for this purpose and make, lot number, etc. always reported.

Procedure: Either three cuts, 4 mm long, or two cuts, 5 mm. long, are made 2 cm. from the end of the slide for the negative pole, and 0 001-0 003 ml. of the heparin solution is applied The reference heparin is applied to one cut Electrophoresis time is 7 minutes at a voltage of 20 V/cm, and temperature 7-11°C. The slide with agar-gel strip is placed immediately in 0.1 per cent Cetavlon for fixation After some hours, or overnight, the slide is placed on filter paper, the agar-gel strip covered with filter-paper and left to dry at 37°C. It is then stained for 5-15 minutes in toluidine blue and rinsed in 1 per cent acetic acid Slides can also be stained for protein with lissamine green as described by van Arkel, Ballieux and Jordan [1] The distance traveled from the point of application, and the optical distance are measured and reported as the ratio to the reference heparin.

Precautions. The preparation of agarose requires standardization for each laboratory. If all the agar is not removed, the background will also be metachromatic. In the Hjerten procedure, residual cetylpyridinium chloride may not be removed and this should be checked with an u.v. absorption spectrophotometer. Drying, staining and washing times will vary with the laboratory conditions, reagents, etc and must be standardized by trial runs. It is desirable to obtain good metachromatic spots with minimal staining of background

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(a) Bleeding Time in the Rabbit by the Method of Roskam and Pauwen[1,2]

This method measures the bleeding time in the rabbit ear. Local physiologic conditions are standardized by observing the hemorrhage under running water of constant temperature and pH, while anatomical variations are standardized by making five cuts in each of four different areas of the ear and calculating the mean of the 20 bleeding times.

Apparatus and Animals: Rabbit board. Water adjusted to pH 6.8-7 0 in a reservoir (Mariotte flask with constant pressure head) The Mariotte flask leads to a glass coil in a thermostatically controlled bath and this to a T-tube bearing a thermometer, ending finally in a Pasteur pipette The latter is held in a clamp to direct a stream of washing fluid on the ear, when required Optimal fluid temperature is 27-30°C. Rabbits must be accustomed to the board and handling. The ears are shaved carefully the day before the test. Other apparatus Sharp scalpel, plastic sheet and container to receive perfusion fluid

Procedure: The rabbit is *not* anesthetized. It is gently fixed on its back on the animal board and the ear immobilized, with the external surface uppermost, by a clamp without causing stasis. The plastic sheet is arranged to receive the flow of fluid With a sharp scalpel, the skin and subcutaneous tissue are cut for about 4 mm., thus cutting small collateral vessels between the central and marginal vessels of the ear. The cut must be made quickly and sharply. A weak jet of wash liquid under feeble pressure is directed on the surrounding tissue some millimeters distant to wash the wound continually. The hemorrhage is observed with the naked eye or with a loupe A stop watch is started when the cut is made and the time noted when the bleeding stops If bleeding does not recur in 30 seconds, the interval is the bleeding time If bleeding recurs, a fresh time is taken

The determination is repeated The ear is divided into four areas I, II, and III are between the central vein and the posterior margin, IV between the central vein and anterior margin, with I, II, and III the central, distal and middle thirds of the ear Five cuts are made in each area of the right ear to give a total of 20 determinations To study the effect of a procedure or drug, this is administered to the animals and, at a suitable time interval, the determinations are repeated on the left ear

The bleeding time is expressed as the mean bleeding time of the 20 determinations (x) Individual bleeding times may be from 20″ to 9′, but the mean bleeding times (x) for normal animals are from 1′ 20″ to 5′ Animals are rejected that show for the right (control) ear, x less than 2′ 10″

CHAPTER I

THE BLEEDING TIME OF THE SKIN

1. Determination of Bleeding Time in Experimental Animals

L. B. JAQUES and G. J. MILLAR

The determination of bleeding time is a useful procedure for the demonstration of the effect of physiologic, pharmacologic and pathologic factors on hemostasis. The concept of measuring the time for bleeding from a standard cut to cease is simple. Difficulties are in standardizing the cut due to the natural anatomical variations in vessel size through the skin, and in standardizing the animal, since this parameter is influenced markedly by various physiologic factors Methods are described for determining this in two common species (the rabbit and the mouse). Borchgrevink and Waaler[1] have described the secondary bleeding test and De Nicola and Candura[2] the measurement of intensity of bleeding in the bleeding time determination. These technics may be used to obtain additional information from the determination of the bleeding time

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[1] Borchgrevink, C F, and Waaler, B A The secondary bleeding time A new method for the differentiation of hemorrhagic diseases Acta med scandinav. *162* 361-374, 1958

[2] De Nicola, P, and Candura, M D Hémostase *I* 113, 1961

stylet with a needle point is employed to cut one of the prominent tail veins. Bleeding time is measured from the moment the blood is seen emerging from the wound until the flow of blood is stopped. Five flows are differentiated: very strong, strong, medium, feeble and very feeble. Whenever arterial flows are obtained, they are easily distinguished by their pulsating character and are excluded from normal venous flow values.

Range of values, 15-220 seconds. Mode value, 30-40 seconds. As the frequency distribution is skewed, log seconds should be used to give a more normal frequency distribution curve. On this basis the mean is 1 655 with an S D of 0.229, equivalent to a mean bleeding time of 45.1 seconds, with ± 3 S D. range of 9.3 to 220 seconds

REFERENCE

[1] Copley, A L., and Lalich, J. J · The experimental production of a hemophilia-like condition in heparinized mice Am. J. Physiol. *135* 547, 1942

or greater than 4′ (15 per cent of animals). Let $x_{\text{left ear}} - x_{\text{right ear}} = d$. Values of x in normal animals are skewed to the right, but d is normally distributed with standard deviation of 20 seconds in 82 animals selected as above. This means that it is justifiable to determine the efficacy of a *hemostatic agent with the paired "t" test on the d values for a series of* rabbits.

The procedure has been modified for use with human subjects.[3]

Precautions are described in the method. The technic and criteria of rejection must be followed exactly. Van Cauwenberge and Jaques have shown that the procedure constitutes a severe stress It might be pointed out that this is one of the few laboratory methods in medical science where sufficient experimental data was collected and adequate statistical research conducted on it and published to ensure the validity of the application of statistical methods. With many methods which lack this background, it is doubtful if even the calculation of standard deviations is on a sound basis of statistical theory.[4]

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3 Pauwen, L J, Roskam, J., Derouaux, G, and Puissant, A Étude statistique du temps de saignement mogen humain Arch internat Pharmacodyn *67*· 390, 1942

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(b) Bleeding Time in the Mouse by the Method of Doettl and Ripke[1]

Equipment. Mouse holder; thermostatically controlled bath; stylet with needle point; chromometer or stop watch; physiologic saline

Procedure The mouse is placed in a brass tube 7.5 cm long, with a perforated cork fitted in one end and the tail inserted in a lucite tail holder closing the brass cage at the opposite end Thus the mouse loses its freedom of movement, but is still in a comfortable position. The brass cylinder is suspended at an angle of approximately 40 degrees by a clamp so that the tail is immersed in a bath of physiologic saline maintained at 37 5°C with a thermostat The saline is changed for each test and, in cases of prolonged bleeding, whenever the blood makes the solution cloudy. A

side, one is connected to an air pump and the other to a manometer. Spiral springs are fitted on glass spikes, suitably placed on the outside of the tube and stopper, to keep the flask closed while maintaining the internal pressure of approximately 20-60 mm. Hg, created by air coming from the air pump The sample of blood is collected in these flasks and protected from air by vaseline. The blood is expelled from the flask gradually by the pressure exerted on the surface of the vaseline (liquid paraffin) by air compressed from the air pump; the pressure is read on the manometer.

Wires in the mercury manometer control the electric connections of the air pump in order to maintain a desired pressure in the top of the special flask. The plastic tube inserted into the vein is left free at its extremity, which provides a means of counting the drops of blood during the experiment. The blood circulates through the preparation at different head pressures in order to maintain a constant rate of flow. To avoid sedimentation, the blood in the special flask is periodically mixed by syringe suction through one of its lateral tubes. The blood is used only once; reoxygenated blood could never be employed

As the central tube almost touches the bottom of the flask, it was possible to expel the last drop of blood through this tube. The pressure in the flask is regulated to a blood flow of 40 to 60 drops per minute as counted in the plastic vein (flow similar to that observed in the leg of normal dogs) To switch easily from one flask containing normal arterial blood to the other with the sample to be tested, a plastic tube adapter (fig 1) is found most convenient Before cutting the skin to initiate the test, it has been found useful to let the blood under test irrigate the preparation for at least 5 minutes

To achieve a smooth and automatic flow through the leg vessels, we have substituted recently a V tube containing acidulated water and two electrodes for the air pump (see page 410) The part of the tube where hydrogen is formed by electrolysis is connected with the flask, hydrogen provides the pressure required for expelling the blood from the tube The generation of hydrogen by electrolysis is controlled by a switch mounted in one arm of a balance, the other arm sustains a cup drained through a needle, in which the venous blood coming from the leg is collected By choosing a convenient needle bore, the level of the blood in the cup and the horizontal line of the balance shaft are adjusted to obtain a constant flow of blood a diminishing flow inclines the balance in one direction making a contact in a mercury cup which energizes the electrolysis unit, while an increase of flow inclines the balance in the opposite direction, cutting off the electrolysis circuit Thus, the automatic control of pressure inside the flask maintains a steady flow of blood in the preparation

CHAPTER II

THE ESTIMATION OF THE HEMOSTATIC EFFECT OF VARIOUS AGENTS

1. *Dog Isolated Hind Leg Preparation Adapted for Studies on Hemostasis*

W. O. CRUZ

Object: A method for experimental studies of hemostasis "in vivo"

Principle: Isolation of an area irrigated by blood samples to be studied is an ideal approach for the in vivo observation of the effect of blood cells, plasma constituents or other substances on spontaneous hemostasis. Under proper conditions, samples of blood irrigating this isolated area are able to hemostatize as in the normal whole organism with the special advantage that the sample of blood is protected from interference by the different organs of the body.

Material Required

1. Normal dogs of medium weight (8 to 10 Kg.), preferably with long and slender legs.

2. Normal citrated arterial blood (0 38 Gm sodium citrate per 100 ml. blood) taken from an anesthetized donor dog and kept under vaseline (liquid paraffin).

3 A powerful tourniquet, preferably made from a transmission or bicycle chain and covered by a rubber tube for skin protection during compression of the leg.

4 *Perfusion apparatus* Two special flasks, one for the sample to be studied and the other to contain normal citrated arterial blood, are immersed in a water bath at 39°C. The flasks consist of a cylindrical tube tapered at the bottom and polished with emory at the open end to accept a ground-glass stopper, through which three glass tubes are passed The center tube is passed to the bottom of the flask; of the two tubes on either

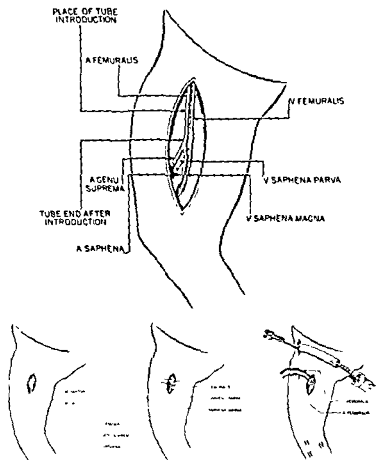


FIG. 2.—Dissecting sequences of dog hind limb preparation

used to press the skin not compressed by the tourniquet. While the tourniquet is being tightened, the leg should be flexed as much as possible to avoid eventual contracture of the extensor muscles To avoid desiccation, cotton soaked in saline was kept over the wound After compression of the leg is achieved, saline is introduced into the arterial plastic tube by means of a syringe with a fine needle, while the limb is submitted to manual massage in order to withdraw from the blood vessels all the blood containing heparin as well as to check the hydrodynamic functioning of the preparation The plastic tube inserted in the artery is then connected with the perfusion apparatus through a plastic tube adapter (fig. 1).

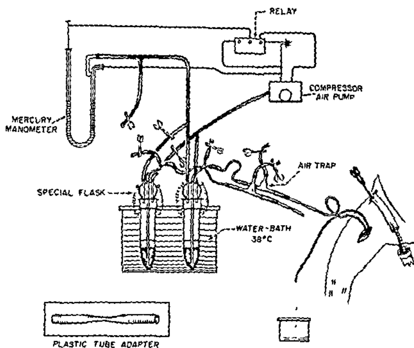


FIG. 1.—Dog hind leg preparation adapted for studies on hemostasis.

Performance of the Test. A much more simplified technic than the one previously described (Cruz and Oliveira, 1958) is now in use in our laboratory. After Nembutal anesthesia, a cannula is inserted into the carotid to follow variations of blood pressure. A cut (no more than 4 cm in length) is then made in the inner part of the thigh in the skin region that covers the area in which the femoral artery gives rise to the genu suprema and continues as the saphena artery (fig. 2). These branches and the corresponding venous vessels (V. femoralis, V saphena magna and V. saphena parva) then were dissected Heparin (300 U per Kg. of body weight) is then administered intravenously and after 10 minutes plastic tubes are inserted in the dissected vessels. A thinner plastic tube (1 mm. internal diameter) is inserted in the femoral artery near the genu suprema artery, care being taken not to introduce it more than 1 to 2 mm from the bifurcation The genu suprema artery is then ligated The venous return circulation is carried out by inserting a plastic tube of a slightly larger internal diameter than that used in the artery in the femoral vein, a little ahead of the venous saphena parva The venous saphena parva is then ligated. The tourniquet chain enclosed by the rubber tube is located in the upper part of the thigh as far as possible from the dissected area and passed through a hole made in the skin fold connecting the leg to the abdomen; a clamp is

6. Too rapid and abundant washing with saline is detrimental to the preparation. A slow flow of saline with gentle manual massage, just enough to wash the blood contained in the leg, is all that is needed.

REFERENCE

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2. Studies on Hemostasis in Vivo (Isolated Rabbit Ear Method of Cruz, Magalhaes, Meis and Dietrich)

W. O. CRUZ

Object of the Method To isolate and perfuse a region under controlled conditions, for studies on skin hemostasis in a small animal.

Principle The ear circulation is isolated and perfused at constant pressure inflow with a citrated blood sample, the ability which to arrest bleeding is then tested by means of bleeding time determinations. All of the results obtained up to now thoroughly confirm those previously described with the use of the dog hind leg preparation [1] The main advantages of this technic are 1 It is simpler to perform; 2, it requires less equipment than dog isolated hind leg, 3 for a great number of laboratories, the rabbit could be a more convenient experimental animal than the dog

Material Required 1 Normal rabbits, as large as possible, in order to facilitate cannulation of the vessels

2. A sturdy forceps, protected with a rubber tube, about 2 cm longer than the ear base

3 Three 10 to 15 ml plastic centrifuge tubes with a rubber stopper through which a large needle and two plastic tubes are inserted. Pressure is applied through the needle, one plastic tube being used for filling the tube with blood and the other as an outlet connected to the preparation by means of a special plastic outfit (shown in fig 1). With such a device, blood perfusing the preparation contacts only plastic surfaces

4 *Perfusion apparatus* Taking into consideration the slight variation of resistance in the rabbit ear circulatory bed, it is possible to use a constant pressure inflow apparatus, instead of a constant flow device as described

Hemostasis Determination: The internal side of the thigh is carefully shaved. With a safety-razor blade, two superficial cuts (5-10 mm.) involving the epidermis, but not the subcutaneous layer, are made far apart, always parallel to the length of the thigh. The blood oozing from the cut is dried every minute by a careful touch of filter paper; the end point of bleeding is taken as the time when a blood spot could not be seen on the paper after this procedure. Thirty minutes was the longest bleeding time recorded; periods longer than that are of little practical interest and considered as infinite bleeding time.

Results: Normal bleeding time taken from 1150 observations in the dog hind leg preparation (citrated arterial blood) gave in 90 per cent of these observations a variation between 5.5 and 11.0 minutes, with an average of 8.0 minutes.

Durability of the preparation tested in 128 cases showed 46 functioning between 1 to 5 hours; 62 between 6 to 8 hours; 20 for more than 8 hours.

Precautions: 1. The hind limb preparation requires a normal nervous influx to work properly. If the regional nerve is severed, if the dog dies in the middle of the test, or even if the condition of the animal is not good (mainly hypotension), then the preparation is not able to hemostatize, even when irrigated with normal arterial blood. 2. With very few exceptions, when a non-hemostatic blood sample irrigates the preparation, it loses its ability to hemostatize, even when hemostatic blood is passing through it afterwards for several hours

Source of Errors

1 If the tourniquet is not tight enough during the entire period of the test, contamination of the leg with blood from the animal is observed, and results are no longer consistent. A second tightening of the tourniquet 10 minutes after the first is advisable due to the softening of tissues squeezed by it.

2. If the tourniquet chain is located too close to the plastic tube insertions, it compresses the vessels during the tightening operation, making the circulation difficult or impossible.

3 During the insertion of the plastic tubes it is not uncommon to produce twisting of the vessels which interferes with the circulation of the blood.

4. Large variations in the blood flow over a long period of time, in some cases, interfere with the control of hemostasis.

5 Care must be taken, when fixing the dog on the table, not to produce stasis in the foot (leather thongs are placed around the fingers, not around the foot) and to avoid pulling the leg so much as to produce an inconvenient rigidity.

ately above the vein, it is convenient to pull the skin slightly to the opposite side, so that there will be no vessels or nerves underneath when the cut is performed. The vein is dissected in a short extension and a thread is passed below it and the artery, no care being taken at this stage to separate them. An extensive "cleaning" of the vessels is then made—a blunt probe and a thin, curved forceps being the best instruments—in such a way that they will be completely free. A thread is passed around the artery and the vessels are then separated. For this step and the following cannulation, a small enlargement lens mounted in a spectacle, and good lighting are essential.

2. *Donor rabbit:* At this stage, or even better simultaneously, a donor rabbit is prepared from which the blood sample will be withdrawn just before use After anesthesia, the neck is shaved, the jugular vein and carotid artery are dissected and cannulated with a bevel-ended plastic tube filled with saline, in which a small piece of soft rubber has been attached so as to facilitate the blood collection. The anticoagulant—10 ml per 100 ml. of blood of a 3.8 per cent sodium citrate solution—is placed into a syringe and the blood withdrawn directly. Care must be taken to wash the cannulae thoroughly with saline to avoid blood coagulation If even a small coagulum is detected, the plastic tube should be removed, cleaned and the cannulation repeated

3 *Ear vessel cannulation This is certainly the most difficult step in the technic and each one must find his own way of doing it properly* Three hundred I.U of heparin per Kg of body weight is injected intravenously (saphenous vein catheter), the cannulation of the ear vessels being started only 10 minutes later. Two pieces of plastic tube, each about 3 cm in length and 1 mm in outer diameter, and with bevelled ends, are used. Larger diameters are preferred whenever possible After some practice no artery forceps will be necessary, the blood oozing will act as a tracer of the vessel cut, especially the vein The vessel is kept distended by pulling the thread, the finger being used as a solid base to fix it Both plastic tubes will be connected during the perfusion with tubes whose inner diameter is about 1 mm, in order to get the lowest possible resistance to blood flow.

4 *Perfusion* Immediately after the ear cannulation is over, 10 ml of citrated arterial blood is collected from the donor rabbit and placed under vaseline in the centrifuge tube. The ear base is then firmly clamped and 3 to 5 ml of warm saline are injected through the arterial cannula to wash the remaining blood The perfusion is started with a pressure of about 40 mm Hg and will be adjusted later in order to produce a flow between 6 and 10 drops (0 2 to 0 4 cc) per minute, equivalent to the normal flow range in the intact rabbit ear To eliminate inconsistent results, the flow must be kept as regular as possible and never below 3 drops per minute,

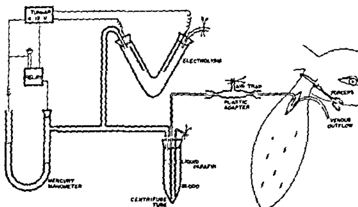


FIG 1 —System used to perfuse the isolated rabbit ear preparation.

in the dog hind leg preparation (p 404) Figure 1 shows the system in which hydrogen from electrolysis of acidulated water produces enough pressure on top of the vaseline (liquid paraffin) layer to force the blood through the ear. The two wires in the mercury manometer work as a switch that controls electrolysis by means of a relay, in such a way that when pressure decreases the electrolysis is switched on, and vice-versa. By moving the free wire up and down it is possible to choose the appropriate pressure for each preparation, generally about 30 to 60 mm. of Hg.

5. A water bath with transparent walls, adjusted to 37°C ; this can be easily improvised with a small glass aquarium.

Performance of the Technic

1. *Ear preparation:* Anesthesia is obtained by sodium pentobarbital intravenously and maintained with sodium thiobarbital, periodically injected through a catheter kept in the saphenous vein. It is better not to make any injection in the lateral ear vein, but if it must be done, the oppposite ear should be used for the preparation

The ear is shaved up to its base with a fine-toothed hair clipper, followed by a razor blade carefully used, with no soap, in order to remove as much hair as possible without hurting the skin. The rabbit is tied in a prone position and the ear fixed in a horizontal support We have been using a plastic prosthetic mold that fits close to the ear, keeping it firmly in position without folding during the whole experiment.

A cut, about 2 cm. in length, is made along the central ear vein, just after its bifurcation. In order to avoid injury to the nerves placed immedi-

from dog arterial plasma[8] to venous blood will convert it to a hemostatic blood. Therefore, it seems to be demonstrated that the isolated rabbit ear is another suitable method for the study of hemostasis "in vivo"

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however, values above 10 or even 20 drops will not affect bleeding time results. Before a sample is tested the saline washing is repeated to avoid any contamination with the previous blood.

5. *Hemostasis determination*· The same technic described in the dog hind leg preparation is used. The cuts are always oriented longitudinally to the length of the ear. Care must be taken to avoid deep cuts which would damage larger vessels, producing profuse oozing of blood Only small vessels should be cut in order to get reproducible results. Each value is the mean of three to six simultaneous determinations made in different regions of the ear. The end point is taken when no further blood spot is detected in the filter paper that is employed to dry up the wound each minute.

Generally, each hemostasis test consists of three successive groups of bleeding time determinations. the first group during perfusion with citrated arterial blood, in order to check if the hemodynamic conditions of the preparation are adequate to stop bleeding of small vessels; the second group with the sample to be tested; and the last one again with arterial blood. Since almost every time the ear is perfused with a non-hemostatic blood the preparation becomes unable to arrest bleeding during the subsequent perfusion with arterial blood, this last group of determinations with arterial blood will measure any after-effect produced by the sample upon the irrigated vascular region, working as a control of the hemostatic ability of the preparation.

Results. Bleeding time performed during perfusion with citrated arterial blood showed, in 124 tests, an average of 6.1 minutes (S D.± 1 6). The durability of the preparation, when continuously irrigated with citrated arterial blood, was 1 to 3 hours in 10 experiments, and greater than 4 hours in 2 experiments

To test the reliability of the method that uses a different skin region in a different animal species, results have been compared to those described with the use of the dog hind leg preparation According to results that will be published in detail elsewhere[2] it was found.

1 The preparation will lose its ability to stop bleeding after rabbit death or ear amputation, confirming the importance of nervous control of the region in which hemostasis is studied;[1] 2 Aeration of arterial blood, in order to decrease its CO_2 content, or its cooling at 1 to 2°C for 30 minutes, will render it non-hemostatic;[3] 3 The same effect occurs after addition of venous plasma[4] or Cohn fraction IV-V from dog arterial plasma;[5] 4. Total platelet removal, by fractional centrifugation, will not change the hemostatic capacity of arterial blood;[6] 5. Venous blood, even after re-oxygenation "in vitro," is non-hemostatic and inhibitory when added to arterial blood;[4] 6. The addition of crude cephalin[7] or Cohn fraction II

CHAPTER III

ESTIMATION OF TYPE AND DEGREE OF HEMORRHAGE-INDUCING FACTORS

1. The Spontaneous Hemorrhage Test

L. B. JAQUES

Object of the Test To evaluate in the *whole* animal the contribution of individual hemostatic factors in the total body economy. In individual investigations, the object of the test can be (a) the measurement of neural, endocrine and other physiologic influences on hemostasis (particularly on the vascular component of hemostasis), (b) the measurement of possible hemorrhagic effects of new drugs, etc ; (c) the measurement of the effectiveness of hemostatic agents

Principle Spontaneous hemorrhage causing 30-100 per cent mortality is produced in animals by simultaneous interference with *two* of the three hemostatic mechanisms, as shown in table 1.

TABLE I

Hemostatic Mechanism Affected	Typical Treatments
1 Blood coagulation (fibrin formation)	Dicumarol, phenylindanedione, heparin
2 Clou hemostatique (platelet plug)	P^{32}, reserpine
3 Vascular integrity	Adrenalectomy, ACTH, stress

*Mortality from Spontaneous Hemorrhage**

Mechanism Affected	1	2	3
1	—	+	+
2	+	—	+
3	+	+	—

* + = 30 — 100 per cent mortality

3. A Method for Evaluating the Hemostatic Effect of Various Agents in Thrombocytopenic Rats and Mice

B. G. FIRKIN, G. ARIMURA and W. J. HARRINGTON

Fifteen Gm. Swiss mice and 50 Gm. Wistar rats are used. Thrombocytopenia is produced by 600 r total body irradiation (250 kv, filtered by ½ mm. Cu and 1 ml. Al at 100 cm.), or by the intraperitoneal injection of a red cell-adsorbed rabbit antimouse or rat platelet serum. Ten to 12 days postirradiation random counts show marked thrombocytopenia Animals injected with antiserum should have overt purpura and extreme thrombocytopenia (platelet counts ranging from 0 to 10,000/cu. mm.) the following day.

Test animals are anesthetized with Nembutal given intraperitoneally, and injected intravenously with either 1 ml. of the appropriate test material for the rat and 0 2 ml. for the mouse, or the corresponding volume of isotonic saline The injection is made into a tail vein or a paw vein. When the injection is completed or, in those animals given a continuous infusion, after an equal "priming" volume has been administered through the infusion set, the animal's tail is severed approximately 1 mm. from its tip with a new razor blade and inserted 1 cm beneath the fluid level in a calibrated tube containing 5 ml. of saline kept at 37°C. in a water bath When the experimental animal is a rat, the blood loss is read off the calibrated scale by measuring the increased volume in the tube at time intervals of 10 minutes, 30 minutes and finally if the animal dies, or when the bleeding has ceased In the mouse a hemoglobin determination is performed when the tail vein is cut and quantitation accomplished either by the above technic or calculated from hemoglobin recovered in the tube. The procedure employed is similar in principle to that described by Hengge (1955).

Normal Range of Values For rats, 0 38 ± 0.11 ml blood lost; for mice, 0.025 ± 0 01 ml.

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TABLE 2. *Dosages of Some Agents Which Have Been Used in the Spontaneous Hemorrhage Test*

Agent	Species	Dose	Action	Mechanism Affected	Parameter for Control Measurements
10% NaCl i p.	rabbit	30 ml for 2 0 Kg + 5 ml for each added 0 5 Kg	stress agent	vascular	
10% NaCl i p	rat	1.75 ml /100 Gm	" "	"	
Formalin	rat	3 75 ml./100 Gm	" "	"	
Insulin	rat	2 8 units/100 Gm.	" "	"	
Dicumarol	rabbit	5 0 mg /Kg	anticoagulant	coagulation	prothrombin time P & P
	rat	2 0 mg /100 Gm rat in 2 5 Gm of food daily for 6 days followed by supplemental food ad lib	"	"	prothrombin time
Phenylindanedione	rabbit	50 mg /Kg single dose	"	"	factor VII
	rabbit	50 mg /Kg + 25 mg /Kg t i d	"	"	prothrombin time
Heparin	rabbit	2000 units intravenously every 8 hours	"	"	coagulation time
P^{32}	rat	4 μc /Gm	thrombocytopenic	platelets	Platelet count
Reserpine	rabbit	0 1 mg /Kg	removes platelet serotonin	platelets	platelet serotonin
ACTH	rabbit	5 mg /Kg	?	vascular	
Adrenalectomy	rat	removed 1 week prior, maintained on 1% NaCl	removal of corticoids	vascular	

Precautions and Sources of Error Many treatments fail to suppress a given mechanism in many animals, e g , rats treated with P^{32} and dicumarol show individuals with a normal platelet count and individuals with only a slight change in prothrombin time These survive and reduce mortality to 30 per cent Increasing dosage will lead to deaths from the agents due to other causes than hemorrhage By hematologic tests, those animals which have responded to both treatments can be identified Chief sources of error are in experimental design In testing a treatment or drug for hemorrhagic action, the combinations shown in table 1 must be tested In testing hemostatic substances, the action of the hemostatic must replace one of the defective hemostatic mechanisms, and since each mechanism involves a series of steps, different procedures produce defects in a given mechanism

Animal Species: The species must be suitably affected by the procedures or agents to be used. With this limitation, any species may be used. We have used rats and rabbits with no apparent differences in results. Where there is considerable individual variation in response to the treatment and this can be distinguished by suitable hematologic tests, a species providing adequate blood samples is desirable. Some dosages for certain procedures are given in table 2

Performance of Tests: Two treatments are selected. One treatment is the parameter being studied The other treatment is considered the basic treatment or constant factor. To be hemorrhagic, the two treatments must affect different hemostatic mechanisms (see table 1 above). When hemostatic agents are being tested, this constitutes the variable and the two *hemorrhagic treatments are constant In selecting treatments, dosages must* be selected to give a marked response in a majority of the animals, and dosage must be adjusted until this is accomplished. This requires preliminary testing and it may be necessary in order to accomplish this to change the dosage schedule (or the animal species to obtain a practicable dosage schedule). In the case of stress procedures, the stress should be sufficiently strong to kill 10 per cent in the control series from the immediate effects of the agent. Treatments are administered in such a way that the maximum effect of each is obtained at the same time Dosages of some treatments used are given in table 2 Experimental animals receive both treatments Groups of control animals receive the single treatments.

These dosages are indicative. Doses adopted must be on basis of being biologically effective without other toxic effects in the animals used. The dose *must be modified for species, genetic strain, age, sex, etc*

Animals after treatment are watched for signs of weakness and hemorrhage. When death occurs, the postmortem examination is performed as soon as possible All body cavities and organs are examined for the presence of free blood The volume of blood or clot present is measured Mucous membranes, liver and viscera are checked for anemia The appearance of blood and/or symptoms of profound anemia are indicative of death from spontaneous hemorrhage Animals should be kept for 3 weeks after treatment Deaths must occur within the period of known effects of the treatments and there should be *negligible mortality in the* groups of control animals.

Results Results are expressed as per cent mortality from spontaneous hemorrhage. Normal range of values is 30-100 per cent Mortality is close to *100 per cent* when the two mechanisms chosen have been effectively suppressed in all animals Significance in reduction of mortality by hemostatic agents can be calculated by conventional statistical methods

Range of Normal Values: Normally urine will not produce a positive benzidine test.

Precautions and Sources of Error: (1) The reagents must be fresh. The benzidine reagent may be stored for 2-3 weeks in the refrigerator. The dilute peroxide solution should be made fresh every few days. (2) The glassware must be chemically clean. Any contamination from blood will produce a false positive test. (3) Catheterized specimens may give a positive benzidine test due to trauma (4) Specimens may be contaminated by red blood cells from the vagina.

2. QUANTITATIVE BENZIDINE TEST: METHOD OF CROSBY AND FURTH[1]

Object of the Test: To determine the amount of blood lost in the urine as Gm of hemoglobin

Principle: Free hemoglobin and all of its derivatives will react with benzindine to form a blue color. The urine specimen is treated with *benzidine and compared in a photoelectric colorimeter with a standard* hemoglobin solution.

Reagents and Apparatus Required: (1) Benzidine base, 1 per cent. One Gm of benzidine base is dissolved in glacial acetic acid and then diluted to 100 ml with distilled water (2) Hydrogen peroxide, 1 per cent. Make fresh every few days from stock superoxol by diluting 1:30. (3) Standard hemoglobin solution Wash 2 ml. of red blood cells with normal saline. Repeat the washing procedure twice Lyse by freezing and thawing. Dilute to approximately 10 Gm per cent with normal saline and determine the exact hemoglobin value by the hemoglobin method used routinely. Prepare a dilute standard by diluting 0 02 ml. of the stock in 10 ml of saline This dilute standard contains 20 mg. of hemoglobin per 100 ml if the stock standard was exactly 10 Gm per cent. (4) Diluent: 10 per cent acetic acid in sulfate-free water. (5) Photoelectric colorimeter.

Performance of the Test: Three tubes are set up for the test. One is for a reagent blank using 0 02 ml of distilled water, the second tube is the standard using 0 02 ml of the dilute hemoglobin standard whereas the third tube is for the test solution and contains 0.02 ml of the urine specimen These three tubes are then treated alike as follows: Add 1 ml of the benzidine solution Mix by swirling Add 1 ml. of the peroxide solution Mix each tube immediately After 20 minutes add 10 ml of the diluent (acetic acid solution). Mix by upending. After 10 minutes read in the photometer at 515 mμ

Calculations: Record the optical densities of the blank and standard on graph paper and connect the points by a line The concentration in Gm of the unknown can be read from the graph

Range of Normal Values Normally there should be no blood present

at different stages of the physiologic process. To be effective, replacement must be made at the place in the chain where the defect has been produced (or later in the chain). If a test is negative, it is advisable to change the design to others involving different steps in the hemostatic mechanism. Interaction of treatments must be watched for. Thus heparin interferes with some effects of stress, while dicumarol shows toxic effects on the cardiovascular system and the liver in addition to its anticoagulant action. Agents and treatments which cause hypertension may introduce a fourth parameter (in addition to the three of table 1) into these experiments

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2. Methods for Estimation of Blood Loss in Body Fluids and Tissues

Described by J. ATWATER and L. M. TOCANTINS

A. *Urine*

1 QUALITATIVE BENZIDINE TEST

Object of the Test To ascertain the presence of blood in urine.

Principle Free hemoglobin and all of its derivatives will react with benzidine to produce a blue color.

Reagents: (1) Saturated solution of benzidine hydrochloride in glacial acetic acid. (2) Hydrogen peroxide, 3 per cent Prepare a fresh solution every few days from stock superoxol diluted 1.10

Performance of the Test. To 2 ml. of the urine sample add 3 ml of the saturated benzidine solution and 1 ml of 3 per cent hydrogen peroxide Mix

Calculations: A blue color indicates the presence of hemoglobin and may be graded from 1 plus to 4 plus according to the degree of blue color developing within 5 minutes No color at the end of this time interval is considered negative.

$$\text{cc. blood lost in 24 hours} = \frac{\text{Gm. Hb/100 cc. urine}}{\text{Gm. Hb/100 cc. blood}} \times \text{cc urine in 24 hours}$$

Range of Normal Values. Normally there should be no blood present.

Precautions and Sources of Error: (1) All urine passed during the collection period must be pooled. (2) Thorough mixing of the pooled specimen is necessary. Blood may form a sediment in the bottle and must be brought into suspension. (3) Large clots may form that cannot be dissolved by mixing and will cause an unavoidable error (4) The diluted specimen must be clear. Any turbidity will invalidate the results (5) Low readings on the Klett photometer are not accurate. Another dilution may be necessary to bring the Klett readings into a more accurate range (at least 20).

B *Feces*

1 QUALITATIVE GUAIAC TEST

Object of the Test To determine the presence of blood in the feces

Principle· Peroxidases present in blood act as a catalyst in the oxidation of the phenolic compound, gum guaiac. Oxygen is provided by hydrogen peroxide and a blue color complex is formed in the presence of blood.

Reagents: (1) Guaiac solution. One Gm. of gum guaiac dissolved in 5 ml of 95 per cent ethanol (2) Glacial acetic acid. (3) Hydrogen peroxide, 3 per cent (4) Filter paper. (5) Applicator stick

Performance of the Test Smear a small quantity of the stool specimen about the size of a pea on a piece of filter paper using an applicator stick. Add 2 drops of glacial acetic acid and mix with the stool. Add 2 drops of the Guaiac solution Add 2 drops of hydrogen peroxide. Mix

Calculations Deep blue color developing in 1 minute is recorded as a 4 plus reaction. No color or only a pale green color after 5 minutes is read as a negative reaction The reaction is graded from 1 plus to 4 plus.

Range of Normal Values· Any reaction above one plus is regarded as indicating significant gastrointestinal bleeding

Precautions and Sources of Error: (1) The test is sensitive, and chemically clean glassware must be used throughout The filter paper should be tested with the reagents to make sure that it does not produce a reaction itself The reagents may be tested in a clean test tube to make sure that they are not contaminated (2) Various lots of gum guaiac may vary in activity It is advisable to test each lot to determine its sensitivity. (3) The gum guaiac test is sensitive but does not require a meat-free diet before testing Nor does medicinal iron interfere with this test (4) Since it is impossible to collect a homogenous specimen, any attempt to quantitate or even semi-quantitate blood loss from the gastrointestinal tract is invalid.

Precautions and Sources of Error: (1) Solutions must be fresh The benzidine solution will keep for 2-3 weeks if refrigerated. The dilute peroxide solution should be made fresh every few days. (2) Glassware must be chemically clean as any contamination from blood will affect the results (3) Crosby and Furth[1] suggest dialysing the urine against saline for 24 hours since certain salts found in urine will precipitate benzidine and may cause turbidity. An adjustment for any change in volume due to the dialysis must be made. The standard hemoglobin solution should be dialysed at the time. (4) Contamination of red blood cells from the vagina or as a result of trauma during catheterization should be guarded against

3. QUANTITATIVE CYANMETHEMOGLOBIN TEST. METHOD OF TOCANTINS[4]

Object of the Test To determine the volume (cc) of blood lost in the urine over a period of 24 hours.

Principle· All urine voided over a 24-hour period is collected, pooled, and the volume recorded. A hemoglobin determination on an aliquot is performed by the cyanmethemoglobin method and compared with the peripheral blood hemoglobin level of the patient. The cc. of blood lost during the 24-hour period is then calculated from the total volume of the collected specimen

Reagents and Apparatus Used· (1) All urine is collected and pooled in a large bottle using a few crystals of thymol as a preservative. (2) Klett Photoelectric Colorimeter calibrated for hemoglobin determinations by the cyanmethemoglobin method using a commercially available standard (3) Diluent. Drabkin's solution. 1 Gm $NaHCO_3$, 52 mg KCN, 198 mg K $Fe(CN)_6$. Dilute to 1 liter with distilled water It will keep in dark brown bottle for 1 month. (4) Cuvettes for Klett (5) Pipettes (10 ml, 1 ml).

Performance of the Test· Collect and mix well all urine voided in 24 hours Measure and record the volume Remove an aliquot Into a Klett cuvette measure 10 ml of the Drabkin diluent Remove 0 4 ml and discard Add 0.4 ml from the urine aliquot and mix Allow to stand 10-20 minutes If not clear, centrifuge at 2000 rpm for 10 minutes to bring down any sediment Read in the Klett and record the hemoglobin value from the calibration chart Do a hemoglobin determination on the patient's blood

Calculations. $\text{Gm Hb/100 cc urine} = \frac{\text{Gm Hb}}{10}$

The urine is diluted 1 25 whereas the standard curve for hemoglobin is based on a dilution of 1.250

Reagents and Performance of the Test: Follow the procedure as described under the section on *Urine*, substituting 0 4 ml of the vomitus specimen for the urine aliquot.

Calculations As previously described

Range of Normal Values. Any blood in vomitus is an abnormal finding

Precautions and Sources of Error: (1) All precautions and sources of error listed previously under the description of this method pertain here also (2) The vomitus specimen may be diluted by mucus and sediment but probably will not cause a very large error, since this method should only be used when large amounts of blood are lost.

D *Menstrual Loss*

1. GRAVIMETRIC METHOD. METHOD OF STEVENSON ET AL.[3]

Object of the Test To determine the amount of blood lost during the menstrual period

Principle. Cellulo-cotton pads are put into large covered jars and weighed After use they are returned to the same jar and re-weighed Assuming 1 Gm of blood is equivalent to 1 cc of blood, the amount of blood lost is determined

Reagents and Apparatus Required. (1) Sufficient Cellulo-cotton pads for the entire menstrual period (2) Large covered jars (3) Scale, accurate to 1 Gm.

Performance of the Test Weigh accurately a supply of cellulo-cotton pads packed in a covered jar Dispense as many jars to the patient as needed for the entire menstrual period Soiled pads are returned to the same jar from which they came and all jars are returned to be re-weighed.

Calculations The difference in weight of the jar with pads before and after use is equivalent to the blood loss for the period One Gm of blood is considered equivalent to 1 cc of blood

Range of Normal Values There is a great variation in the amount of blood lost during any menstrual period by normal individuals Using the procedure outlined here, Stevenson and co-workers[3] report the following values Range 2-165 Gm with a mean variation of 48 5 and a standard deviation of 31 4

Precautions and Sources of Error (1) Unavoidable errors include (a) loss before donning of pad, (b) loss during bathing, (c) evaporation while wearing the pad, (d) contaminants weighed with pad, such as powder. (2) Storage evaporation is controlled by the use of covered jars.

2. QUALITATIVE BENZIDINE TEST

Object of the Test: To determine the presence of blood in the feces.

Principle: Free hemoglobin and all of its derivatives will react with benzidine to produce a blue color.

Reagents and Performance of the Test: A suspension of stool in a small amount of water is substituted for urine in the qualitative benzidine test as described in the section on *urine*.

Calculations and Range of Normal: The benzidine test is more sensitive than the guaiac test for occult blood in feces and should be interpreted with reservations.

Precautions and Sources of Error: (1) All precautions listed under this procedure in the section on *Urine* must be observed here. (2) The extreme sensitivity of benzidine in this procedure requires that the patient be placed on a meat-free diet for several days before collecting the specimen of stool to be tested

C. *Vomitus*

1 QUALITATIVE BENZIDINE TEST

Object of the Test: To determine the presence of blood in the vomitus.

Principle: Benzidine will react with free hemoglobin and all of its derivatives to produce a blue color

Reagents and Performance of the Test: A qualitative benzidine test is performed as described in the section on *Urine* substituting 2 ml. of vomitus for the urine aliquot

Calculations. Any blue color is recorded as a positive test

Range of Normal Values Normally, the benzidine test should be negative

Precautions and Sources of Error (1) All precautions mentioned previously under the section on *Urine* must be observed. (2) Fresh blood in vomitus may be suspected by its bright red color; however, older specimens may be a dark coffee color (3) Mucus or heavy sediment should be removed from the vomitus by filtering through gauze

2. QUANTITATIVE CYANMETHEMOGLOBIN TEST

Object of the Test To determine the amount of blood lost in the vomitus.

Principle Hemoglobin is converted to methemoglobin and then to cyanmethemoglobin with the use of Drabkin's diluent (see p. 000). The standard cyanmethemoglobin curve is used as a basis for determining the amount of blood lost in the vomitus.

trated ammonium hydroxide (5) Oxyhemoglobin determination on patient pre-operatively. (6) Graduated cylinder

Performance of the Test: An oxyhemoglobin determination is done on the patient before the operation by diluting 1 ml of whole blood (collected accurately in a syringe) to 100 ml. with distilled water. A drop of concentrated ammonium hydroxide is added to a 5 ml aliquot before reading to convert the lyzed blood to oxyhemoglobin. The hemoglobin is recorded in mg. from a previously plotted calibration curve After the operation is completed another hemoglobin determination is made from the tub in which all sponges and linens have been agitated until clean, and all instruments have been washed free of blood Any suctioned blood is pooled with these washings and the total volume is measured. An aliquot of 5 cc. is centrifuged and a drop of concentrated ammonium hydroxide solution is added. The mg of hemoglobin in the washings is then interpolated from the calibration curve

Calculations. Total blood loss in cc is derived by applying the following formula:

$$100 \times \frac{\text{mg Hb in sample}}{\text{mg Hb pre-op.}} \times \text{total volume of washings in cc.}$$

Range of Normal Values: See "Gravimetric Method."

Precautions and Sources of Error. This is a modification of the method of Gatch and Little,[7] who used the acid-hematin procedure instead of oxyhemoglobin for the determination of the hemoglobin values Every effort must be made to extract all blood from sponges, linen and instruments A clear solution is a necessity for accurate results and any lint or tiny clots must be removed from the aliquot that is converted to oxyhemoglobin and read in the colorimeter Any clots present should be broken up and lyzed or a low value will result.

3 CONDUCTIVITY METHOD METHOD OF LEVEEN ET AL.[8]

Object of the Test Continuous determination of blood loss during an operative procedure

Principle Blood acts as an electrolyte of fairly constant composition. By using a wheatstone bridge arrangement connected to a tub of water into which all blood lost during the operation is pooled, a continuous direct reading dial registers the amount of blood lost at any time.

Reagents and Apparatus Required (1) A "Blood Loss Monitor" is available from Industrial Instruments, Inc, Cedar Grove, N. J.—Model BLM-1 (2) Anti-foam solution of a non-electrolyte type (Critical SP-80 Anti-foam solution) (3) Tap, distilled or deionized water for filling the tub

E. *Loss during Surgical Operation*

1. GRAVIMETRIC METHOD. METHOD OF BARONOFSKY[4]

Object of the Test: To determine the amount of blood lost at any point during the operative procedure.

Principle. It has been determined that sponges are remarkably uniform in dry weight and come from the autoclave in a fairly constant degree of dryness. The difference in weight of the wet sponges is, therefore, equal to the blood loss at any point.

Reagents and Apparatus Used: (1) A scale accurate to 1 Gm. (2) Sponges of a size that have had their weight determined and recorded after autoclaving.

Performance of the Test: At frequent intervals during the operative procedure the weight of the wet sponges is recorded in Gm.

Calculations: The difference in weight of the sponges before and after use is recorded as the Gm of blood lost up to that time. One Gm. of blood is considered equivalent to 1 cc. of blood lost

Range of Normal Values: The type of operation is the most significant factor in blood loss at operation. Within any specific category, however, the length of time involved will contribute an important factor. The amount and type of hemostatic control by the surgeon cannot be overlooked. Baronofsky et al[4] report gravimetric blood loss varying from an average of 25.8 cc in appendectomy to an average of 1399 cc. in pneumonectomy and lobectomy

Precautions and Sources of Error Weighing should be done at frequent intervals so that evaporation does not become a factor Serous fluid or pleural fluid does not interfere with the determination since normally it will only average 2-4 Gm. per cent The scale must be accurate to 1 Gm. Blood lost by being suctioned off instead of absorbed by sponges may account for a considerable error at times The technic will give a reasonable estimate of blood loss at any time during the operative procedure, however.

2 COLORIMETRIC METHOD. MODIFICATION OF METHOD OF GATCH ET AL[5]

Object of the Test To determine the amount of blood lost during an operative procedure

Principle. The oxyhemoglobin value of the washings from all instruments, sponges, linen and suction procedures is determined and compared with the patient's preoperative hemoglobin

Reagents and Apparatus Used (1) Large tub with some type of agitator to extract blood from sponges, linens, etc (2) Distilled water (3) A photoelectric colorimeter calibrated for oxyhemoglobin (4) Concen-

SECTION C

THROMBOSIS

METHODS FOR THE EVALUATION OF THROMBOSIS IN VIVO

Performance of the Test: The tub is filled with 24,000 cc. of water. The switch for the electronic equipment is turned on, and about 3 or 4 minutes are allowed for the warming-up period All sponges or linens are added to a wire basket suspended in the tub and blood is extracted by means of a mechanical agitator. A special device is available to deposit *suctioned blood* directly into the tub.

Calculations: Two dials are present which register either small amounts of blood loss, as is expected in pediatric surgery, or for the larger amounts of blood loss in adult surgery. The dials measure increments of 20 cc. of blood but interpolation to 5 cc. is easily made.

Range of Normal Values: See "Gravimetric Method."

Precautions and Sources of Error: The tub should be filled with distilled or deionized water if the original readings on the dial register 500 cc.

Blood Loss with Tap Water. Anything less than a reading of 450 cc. for tap water may be subtracted from the dial readings as blood is added to the tub. Pleural or peritoneal cavity fluids should be suctioned off separately and not added to the tub, because they also act as electrolytes. Salt solutions used for irrigations will be indicated as blood loss, if added to the tub. Instead of salt solutions it is suggested that a 2 5 per cent glucose solution be used for irrigations A correction must be made for large amounts of irrigation solutions added to the tub. If the use of 1000 cc. is anticipated, only 24,000 cc. of water should be added to the tub originally. Proteins from hemoglobin or plasma tend to depress the conductance and, therefore, anemia will produce small errors; however, as transfusion corrects the anemia, the conductance returns to its normal state also.

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The in Vitro Test. The in vitro test has the advantage of needing only one sample of blood and is thus more acceptable to the patient. In the original test[2] it was assumed that the accelerated coagulability of blood following operation, inflammatory conditions and enforced bed rest was due to an increase in tissue thromboplastin The accelerated coagulability of blood was more easily recognized in the presence of small amounts of heparin. Research during recent years would cause many investigators to question the original assumption Nevertheless the test is still useful as an indirect means of detecting hypercoagulable tendencies, and as a guide for heparin dosage.

Procedure Saline solutions containing the following concentrations of heparin are prepared 0 00, 0.20, 0 40, 0 60, 0.80, 1.00, 1.20 and 1 50 units per ml One-half ml of each heparin solution is placed in a separate clotting tube The tubes are then placed in a constant temperature bath (good results can be obtained at room temperature using a 2 inch deep wooden block in place of a water bath). Either clean dry tubes or siliconed tubes can be used provided all the tubes in any one test are similar Exact sizing of the tubes is important Ten ml of whole blood are withdrawn from the arm vein of the patient with a silicone-treated syringe and needle. One ml is placed in each of the tubes and the tubes are agitated gently to insure mixing The clotting time of each tube is determined to the nearest half-minute by examining it every 30 seconds The end point is when the blood no longer flows when the tube is tilted at 75° angle. A graph is then prepared plotting clotting times in minutes against heparin concentration The slope of the line thus produced indicates the sensitivity of the blood to heparin The less the sensitivity to heparin, the less the slope The results of such a test are more qualitative than quantitative in nature The test therefore is most useful in following changes in the coagulability of a patient from day to day rather than comparing different patients[3]

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CHAPTER I

ANTICOAGULANTS

1. The Heparin Tolerance Test

F. C. MONKHOUSE

Objective: To provide an estimate of the coagulability of circulating blood. To determine the advisability of anticoagulant treatment and to aid in establishing a suitable heparin dosage clinically.

The in Vivo Test: There is considerable variation in the response of different individuals (or the same individual under different circumstances) to heparin. To aid in the adjustment of a safe but effective dose of heparin, de Takats[1] developed an in vivo heparin tolerance test. This was a simple test designed to measure the patients sensitivity to heparin

Procedure Fifteen mg. of heparin (1500 units) are injected intravenously. Whole blood coagulation is determined before and 10, 20, 30, 40 and 60 minutes after injection Blood should be withdrawn into a siliconed syringe. Coagulation time is best determined at 37°C. in dry glass tubes using a two-tube Lee and White technic. Three to 5 ml. of blood are withdrawn One ml. is placed in each of two tubes and the remainder discarded. The tubes are allowed to set for 4 minutes, then one tube is tipped gently to 75° angle every 30 seconds until the blood no longer flows The second tube is treated similarly. The clotting time is recorded as that of the second tube, which, of course, should be the one to receive the blood last taken from the patient The results should then be graphed with coagulation times showing in the ordinate and time of sampling on the absicca Normally, the coagulation will be doubled at 10 minutes and remain prolonged up to 30 or 40 minutes. In the hyporeactors the increase in coagulation time will be less marked, the duration may be somewhat less but not necessarily so In the hyperreactors the increase is much greater and usually the effect is more prolonged

The difficulty in measuring clotting times accurately is the main drawback to the method. Meticulous care in sampling and handling of tubes is required.

Fibrinolytic agents effective against blood clots formed by procoagulants in vitro should be effective against blood clots formed by these same agents in isolated venous segments. Perhaps they would not be effective against intravascular accumulations of platelets, white cells, or red cells formed by virtue of blood flowing past a site of injury. The effectiveness of experimental therapeutic measures may depend in large measure upon the method of thrombus inducement. Therefore it must not be assumed that these measures would be equally effective against thrombi formed by other and different sets of mechanisms.[11] Injections of thrombosing substances into isolated vascular segments usually produce occlusive masses within a matter of minutes. Time and blood flow as necessary factors in the development or dissolution of thrombi are practically eliminated by these methods.

Many of the precautions discussed above also apply to methods utilizing ligatures and clamps. John Hunter[12] in the 18th century observed, "that the blood of a fish, which had the actions of life stopped for three days, and was supposed to be dead, did not coagulate in the vessels; but, upon being exposed, or extravasated, soon coagulated." Wessler[13] isolated venous segments by double ligation and found clots within an average time of 90 minutes after ligation Ligating and clamping vessels have not been satisfactory methods to produce thrombi experimentally because they lack consistency and reproducibility. These methods have usually been combined with methods of mechanical trauma, injections of thrombosing substances, and perivascular applications of materials damaging to the vessel wall[1] Although these additional measures increase the incidence of experimental thrombosis, they are not without objection For instance, the use of perivascular applications of fixing agents, such as formalin or osmium tetroxide, may reduce or even eliminate certain metabolic activities necessary in the development or dissolution of thrombi These activities include the role of the cells of the vessel wall, especially the endothelial cells, the role of living viable blood elments, such as white cells and platelets, and the role of bacteria in the stages of thrombogenesis or thrombolysis[11]

The role of trauma in the etiology of thrombosis is difficult to assess I ask myself, "How severe is an injury? Are cells of the blood vessel walls 'injured' in diabetes mellitus, a condition predisposing to thrombosis? In any condition of circulatory failure or slowing of the blood, are the vessel walls 'injured' by anoxia?" Burns, electric currents applied directly, injections of thrombosing substances, injections of sclerosing agents, perivascular applications of fixing agents or sclerosing agents, intravascular foreign bodies, ligatures and clamps applied to vessels, vascular surgery, and direct mechanical trauma to the endothelium or to the whole vessel

CHAPTER II

EXPERIMENTAL PRODUCTION OF THROMBOSIS

1. *Methods for the in Vivo Study of Thrombosis*

R. L. HENRY

Experimental studies on thrombosis are desirable from the standpoints of elucidating the etiology and pathogenesis of thrombosis and its prevention or cure. To produce thrombi experimentally, assumptions must be made concerning etiology. It is virtually impossible to select one basic underlying causative factor for thrombosis from the hundreds of different experimental methods of inducement.[1] Experimental thrombi must be produced in a consistent and reproducible manner to study the effects of preventive or curative measures The purposes of this section are to orient the reader on the methods available to experimentally induce thrombi and to list some considerations to be made before selecting a particular method better suited to the investigation at hand

The majority of methods used to experimentally induce thrombi fall into several categories: (1) injections of thrombosing substances; (2) mechanical trauma, (3) ligatures and clamps; (4) in vitro procedures For a more extensive list the reader is referred to a recent annotated bibliography by Henry (1962) [1]

The mechanism (or mechanisms) producing thrombi by intravascular injections of thrombosing substances is not clear Injections of procoagulants such as thrombin[2,3] or thromboplastin[4,5] are assumed to induce thrombi by their direct effects on the blood It must not be assumed that this is the main or the only means by which all thrombi form Several precautions are noteworthy at this point Blood flow is eliminated as a possible thrombogenic or thrombolytic factor when a blood clot is formed by injecting a procoagulant into an isolated vessel segment,[2,6-9] It is impossible for the blood to bring elements such as platelets and white cells, known to increase with time in early thrombus formation in vivo,[10] into a vessel segment which has been isolated by ligatures or clamps.

Fibrinolytic agents effective against blood clots formed by procoagulants in vitro should be effective against blood clots formed by these same agents in isolated venous segments. Perhaps they would not be effective against intravascular accumulations of platelets, white cells, or red cells formed by virtue of blood flowing past a site of injury The effectiveness of experimental therapeutic measures may depend in large measure upon the method of thrombus inducement. Therefore it must not be assumed that these measures would be equally effective against thrombi formed by other and different sets of mechanisms.[11] Injections of thrombosing substances into isolated vascular segments usually produce occlusive masses within a matter of minutes Time and blood flow as necessary factors in the development or dissolution of thrombi are practically eliminated by these methods

Many of the precautions discussed above also apply to methods utilizing ligatures and clamps John Hunter[12] in the 18th century observed, "that the blood of a fish, which had the actions of life stopped for three days, and was supposed to be dead, did not coagulate in the vessels, but, upon being exposed, or extravasated, soon coagulated" Wessler[13] isolated venous segments by double ligation and found clots within an average time of 90 minutes after ligation Ligating and clamping vessels have not been satisfactory methods to produce thrombi experimentally because they lack consistency and reproducibility These methods have usually been combined with methods of mechanical trauma, injections of thrombosing substances, and perivascular applications of materials damaging to the vessel wall[1] Although these additional measures increase the incidence of experimental thrombosis, they are not without objection For instance, the use of perivascular applications of fixing agents, such as formalin or osmium tetroxide, may reduce or even eliminate certain metabolic activities necessary in the development or dissolution of thrombi These activities include the role of the cells of the vessel wall, especially the endothelial cells, the role of living viable blood elments, such as white cells and platelets, and the role of bacteria in the stages of thrombogenesis or thrombolysis[11]

The role of trauma in the etiology of thrombosis is difficult to assess. I ask myself, "How severe is an injury? Are cells of the blood vessel walls 'injured' in diabetes mellitus, a condition predisposing to thrombosis? In any condition of circulatory failure or slowing of the blood, are the vessel walls 'injured' by anoxia?" Burns, electric currents applied directly, injections of thrombosing substances, injections of sclerosing agents, perivascular applications of fixing agents or sclerosing agents, intravascular foreign bodies, ligatures and clamps applied to vessels, vascular surgery, and direct mechanical trauma to the endothelium or to the whole vessel

can all theoretically be reduced to vascular injury. Yet, it has been stated that vascular injury alone cannot induce thrombosis.[14,15] Slowing of the blood flow past the site of vascular injury may be a necessary thrombogenic factor. Perhaps procoagulants are introduced from the vessel wall at a site of injury. In any case, these considerations lead to sets of mechanisms in the inducement of thrombi. Combinations of factors may occur in diverse order and in each case lead to an intravascular accumulation called a thrombus. No given method is the better method for inducing experimental thrombi. The conclusions and concepts adduced from different methods vary according to the particular method used and apply only to thrombi induced by that method and closely similar methods.

A further caution in the study of experimental thrombosis is in regard to the method used to find the thrombi produced. Opening a blood vessel to allow the fluid parts to drain off or otherwise rinsing a vessel segment may lead to loss of small or weakly adherent thrombi. Quick-freezing and subsequent freeze-substitution is a satisfactory means of examining experimentally thrombosed vessels eliminating the chance of overlooking any thrombus formed at the site of inducement.[10]

Time is an important factor when determining the incidence of thrombosis from any given method of inducement Thrombogenic and thrombolytic processes occur simultaneously from the time of inducement Which process is occurring at a more rapid rate will determine the size and presence or absence of a thrombus in time The absence of a thrombus does not always mean that a thrombus did not form It may have formed and was subsequently dissolved by the animal's own lytic mechanisms The time of greatest incidence of thrombi present is not an arbitrary value but varies with the particular experimental method of thrombus inducement Electrocoagulation or certain procoagulants, if severe enough, consistently induce occlusive blood clots in minutes or even seconds The highest incidence of thrombosis from such experimental stimuli as mechanical trauma, foreign bodies inserted intravascularly, or partial ligations may require hours or even days to develop Whatever experimental method of thrombus inducement is chosen, time must be given careful consideration in analyzing the effectiveness of that particular method For these purposes the following method of quick-freezing and freeze-substitution fixation is presented as a satisfactory means of preparing blood vessels for determining the incidence of thrombi produced experimentally, and for their histologic analysis

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2. Study of Thrombosed Blood Vessels by Quick-Freezing and Freeze-Substitution Fixation

R. L. HENRY

Object of the Method. A method designed to halt all living processes at a designated time following thrombus inducement in blood vessels of experimental animals The method conserves three-dimensional architecture as it was in vivo and reduces the chance of losing or overlooking thrombi produced

Principle Very rapid quick-freezing reduces inevitable ice crystals to submicroscopic size resulting in good histologic structure at relatively high magnifications. This structure is maintained through freeze-substitution by replacing the tissue water with a fixative of choice while holding the tissue in the frozen state. Segments of blood vessels containing thrombi and blood can then be serial-sectioned using routine histologic technics

Reagents and Apparatus Required: (a) Liquid nitrogen and an insulated liquid nitrogen storage tank. (b) A 4-liter DeWar flask or a similar insulated container capable of handling liquid nitrogen. (c) Fixative reagent of choice. The fixative must remain fluid at the freeze-substitution temperature. (d) A cryostat or cold box in which blocks of frozen tissue can be removed for freeze-substitution. The cryostat should be capable of maintaining a temperature of −40°C to −50°C. (e) Dry ice and an insulated chamber for dry ice (f) Routine histologic reagents and equipment (e.g., microtome, paraffin oven, heating plate, staining dishes, and mounting instruments).

Steps in Procedure: The following procedure will be described using the rat as the experimental animal. Any experimental animal can be used although amphibian tissues in general are not preserved as well as mammalian tissues during freezing[1] The choice of the dog and other large animals will require an adaptation of the quick-freezing procedure.

Following inducement of thrombosis in the vascular site of choice, all subsequent procedures are directed toward preservation of that vascular segment. Not all areas of an animal freeze equally well without damage when the entire animal is quick-frozen In general the more superficial structures will be well preserved whereas the deeper structures may show severe ice crystal damage If a blood vessel, such as the external jugular vein of the rat, is chosen, it is better to quick-freeze the cranial one-half of the animal first to allow dissipation of heat to the caudal one-half The caudal one-half should then be frozen or heat will be conducted from it, and thus thaw the cranial one-half resulting in ice crystal damage

The rate of quick-freezing is important[1] Liquid nitrogen (−205°C.) is placed in the DeWar flask and the anesthetized animal is plunged directly into the supercool fluid Liquid propane or liquid oxygen may also be used but they involve some danger to the user In any case, the freezing medium should be at least −175°C for quick-freezing whole animals or large pieces of tissue

Once frozen, the animal is transferred to a cold chamber (a cryostat capable of maintaining −50°C is satisfactory) where the experimental vascular segment is chipped out Ordinary hobby shop wood carving chisels are excellent for frozen dissection. Care should be taken to carve contiguous tissues away from the piece to be saved before it is finally

chipped loose. More refined carving of the selected piece can then be performed. The piece should be as small as possible to insure rapid freeze-substitution

The experimental vascular segment is now transferred to a fixative solution previously chilled to at least −50°C. on dry ice. One per cent mercuric chloride in absolute alcohol is satisfactory for most histologic purposes.[2] The amount of fluid fixative should equal 100 times the tissue blocks by volume Bottles containing fixative and tissue are then transferred to a dry ice chamber where water in the tissue is replaced by fixative Since substitution occurs at the rate of approximately 0 5 mm. per day in this system, the number of days for freeze-substitution can be calculated from the size of the tissue block. For example, a block of tissue 2 mm. in its smallest dimension will require at least 4 days of freeze-substitution. They may be left any period longer than the required time.

The tissues are brought to room temperature in the same fixative mixture. They are then washed with two one-half hour changes of absolute alcohol, cleared for one-half hour in xylol or dioxane, and embedded in paraffin Two one-half hour changes in paraffins with melting points ranging from 50-52°C and 60-62°C and final embedding in 60-62°C. paraffin have been found satisfactory. These time limits vary with the size of the tissue block and are ascertained only after experience The above schedule applies to tissue blocks up to 5 mm in greatest dimension. Routine blocking, cutting, mounting, and staining can now be performed

Limitations and Usefulness of the Method: The method of quick-freezing will have to be adapted for larger animals such as the dog Obviously, it would be difficult to freeze the entire animal In order to freeze blood vessels in situ a retainer area for the quenching medium will have to be formed around the experimental vascular segment It is possible to choose blood vessels which, when exposed, lie in the floor of a natural anatomical fossa The quenching fluid should be poured directly onto the vessel within the retaining area The experimental vascular segment should be rapidly chipped out and transferred to a cold chamber for more refined carving before thawing occurs

Any fixative can be used for freeze-substitution provided it remains fluid at the subzero temperature chosen for freeze-substitution. Since tissues containing water will form ice crystals when thawed above −10°C. they should never be allowed to reach a higher temperature until freeze-substitution is complete Freeze-substitution occurs more rapidly at higher subzero temperatures, however, more time at colder temperatures is preferable in most cases

Quick-freezing, if rapid enough, halts all living processes almost instantaneously. Relationships of the blood, the thrombus, and the vessel wall are preserved in the original in vivo condition. These relationships *can be viewed in a three-dimensional way through the study of serial* sections of the vascular segment. Furthermore, since it is possible to prepare cross-sections of the column of blood in the vessel lumen adjacent *to the thrombus, comparisons of the number of specific blood cells in the* thrombus to the number found in an equal area in the column of blood can be made in the same tissue section. When these comparisons are made for different *thrombi frozen at increasing time intervals after induce*ment, an increase or decrease in specific blood cells with time of thrombus formation or dissolution can be determined.

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3. *Methods for Production of Platelet Thrombosis in Animals*

L. B. JAQUES and J. ASHWIN

A. *Vein Crushing in Dogs*

The object of the test is to produce a platelet thrombus in veins which can be used to determine effectiveness of (a) anticoagulants in preventing formation of thrombi, and (b) agents causing resolution of thrombi The *principle* of the test is to traumatize the endothelium of a vein and prevent immediate clotting by a small dose of heparin so that the vessel is gradually filled with a platelet thrombus

Materials Dogs of 8-12 Kg, Nembutal linen thread, Barbour's No 25, Surgical instruments

Procedure· *Under barbiturate anesthesia, and with aseptic technic, the* saphenous or brachial vein of a dog is exposed for a distance of 35 mm A plain, straight needle (non-cutting), carrying linen thread, is passed up the vessel for 20 mm Hemostat (5½") clamps are placed on the vessel at the puncture holes A third hemostat is then used to crush the 18 mm. of

vein between the clamps The crushing is repeated twice, rotating the vein so that the crushing is performed in three planes. The clamps are removed. Hemorrhage is controlled by gentle pressure. Heparin (70 U./Kg.) is injected into another superficial vein and the incision closed. The incision is reopened after 6 hours or 72 hours or 3 weeks. The gross appearance of the vein is recorded, clamps placed on the vessel, and the segment of vein removed. Flow of blood and appearance of the cut end are recorded and after fixation, cutting and staining with hematoxylin-eosin, the histologic appearance of the vein wall and contents are recorded.

To test the ability of agents to prevent thrombus formation, these are administered to act for the first 8 hours and the vein examined at 6 hours. To test for prevention and healing, the agents are given over 48 hours and the examination made at 72 hours. To test for agents causing resolution of thrombi, the agents are given after 6 hours and the veins examined either at 72 hours or 3 weeks. Results are expressed as per cent incidence of thrombosis Untreated animals show an incidence of 60 per cent thrombosis. The thread may be removed from the vein after crushing without significant change in results but this increases the difficulty of controlling bleeding, particularly with heparinoids

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B. Jugular Vein Exposure in Rats[1,2]

This method makes possible the rapid and statistical assessment of factors which may increase or decrease thrombosis Exposure of the rat jugular vein causes platelets to stick on the inner wall of the vein Histologic studies show that sticking begins after 10 minutes Thrombosis is complete after 60-100 minutes and histologically resemble clinical cases

Reagents and Apparatus Required. Ether, scalpel, forceps, probe, wound clips, 10 per cent formalin in 65 per cent methanol; rats of either sex, 100-150 Gm. At least 20 rats should be used in each series

Procedure The rat is anesthetized with ether, and the area sponged with dressing alcohol A skin incision is made with scalpel cephalad of the clavicle 1 cm and slightly to right of the midline The right jugular vein is cleared and 5 drops of formalin solution are dropped on the vein The

Quick-freezing, if rapid enough, halts all living processes almost instantaneously. Relationships of the blood, the thrombus, and the vessel wall are preserved in the original in vivo condition These relationships can be viewed in a three-dimensional way through the study of serial sections of the vascular segment. Furthermore, since it is possible to prepare cross-sections of the column of blood in the vessel lumen adjacent to the thrombus, comparisons of the number of specific blood cells in the thrombus to the number found in an equal area in the column of blood can be made in the same tissue section. When these comparisons are made for different thrombi frozen at increasing time intervals after inducement, an increase or decrease in specific blood cells with time of thrombus formation or dissolution can be determined.

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Procedure: The rat is anesthetized with ether, and the area sponged with dressing alcohol. A skin incision is made with scalpel cephalad of the clavicle 1 cm. and slightly to right of the midline. The right jugular vein is cleared and 5 drops of formalin solution are dropped on the vein. The

wound is closed with a single clip and the test solution is injected in the sublingual vein.[3] The rat is returned to its cage. Five hours later the animal is again anesthetized and the clip removed. The vein is examined for clots which appear as black or black and white plugs within the lumen.

Results are expressed as percentage incidence of thrombosis. Significance of differences between means of groups of series may be determined by means of the 't' test.

The incidence of thrombosis varies with the size of animals so that normal control series should be run with every experiment Mean values with standard deviation are about 60 ± 7 per cent. The incidence of thrombosis after various treatments ranges from 0-100 per cent.

Precautions and Sources of Error: The incidence of thrombosis shows good agreement between different examiners and between gross and microscopic examination. Very small, white mural patches are ignored, there should be a definite plug. Animals of the same colony, age and sex should always be used. Results are more consistent with the use of formalin solution and particularly if it is freshly made in 100 ml. amounts.

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C. Demonstration of Platelet Thrombi[1]

The object of the test is to determine the effect of factors (such as anticoagulants) on formation of platelet thrombi by direct visual observation. A glass cell is placed as a shunt between a suitable artery and vein and formation of thrombi observed

The *cell* is shown in figure 1. The cannulae are of 2 5 mm pyrex tubing 3″ in length The cell is 2 cm in diameter and has a scratch on the upper inner surface of the cell

Animals are anesthetized and the cell inserted Results are recorded for direct visual observation and also time for filling of the cell with white thrombi Platelet thrombi form on the scratch, then at the periphery until the cell is filled. Solandt, Nasim and Best[2] found prevention of formation of platelet thrombi in the cell did not parallel concentration of heparin in the circulation.

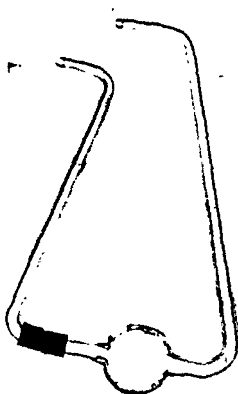


Fig 1

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4. Experimental Production of Thrombosis by Administration of Serum

S. WESSLER

Object of Test· This test produces standard thrombi in the large veins and arteries of animals and can be used to study the relation of clotting factors to the phenomenon of in vivo coagulation. With minor modifications the technic may also be employed to investigate the spontaneous fate of thromboemboli and their reaction to anticoagulant and lytic agents as well as the effects on an organ of thrombotic obstruction of its blood supply.

Principle of Test: The method is based on the observation that the systemic infusion of glass-contacted mammalian serum induces in recipient animals non-adherent thrombi of known histologic composition only in areas of retarded blood flow. This thrombogenic activity in serum has been termed Serum Thrombotic Acclerator (STA). It arises early in the elaboration of intrinsic thromboplastin and is distinct from thrombin and tissue thromboplastin. Whether STA activity is a discrete clotting factor or a reaction product formed during the early stages of coagulation is unknown at the present time. The phenomenon of thrombosis is quantitatively related to the amount of serum infused and has exhibited no species specificity among mammals.

Reagents and Apparatus Required (for the production of rabbit vein thrombosis by human serum): Human serum, male rabbits, surgical instruments and suture material, syringes, needles, petri dishes, stop watch, water bath, 6 per cent sodium pentobarbital, 0.85 per cent sodium chloride, and 5 per cent sodium citrate

Steps in Performance of Test Human serum is prepared from blood obtained from healthy donors by primary venepuncture Freshly drawn blood, transferred from uncoated glass syringes to uncoated centrifuge tubes, is allowed to clot for 2 hours at room temperature and for an additional 18 hours at 4°C The serum is then separated from the formed elements by centrifugation at 2500 g at 4°C for 20 minutes Serum thus prepared is essentially devoid of thrombic activity and may be infused immediately or stored in aliquots at −20°C for periods as long as 12 months

Rabbits are anesthetized with intravenous sodium pentobarbital (0 73 ml per Kg.) A 1-2 cm length of external jugular vein is then freed from its surrounding structures and its tributaries ligated Human serum (1 32 ml per Kg diluted with sodium chloride solution to a fixed volume of 5 ml and warmed to 37°C for 15 minutes immediately prior

to infusion) is injected in 15 seconds into a contralateral ear vein. Within 15 seconds after completion of the infusion, the previously exposed jugular vein is gently isolated with silk or cotton ligatures. The isolated jugular vein segment is allowed to remain in situ for 10 minutes The segment is then removed from the animal, its contents emptied into a petri dish containing 30 ml. of sodium citrate solution, and the contents of the dish examined with the aid of a suffused transmitted light for the presence of gross thrombosis.

Manner of Expression of Results: The amount of thrombus formed may be scored on a scale of 0 to 4; a score of 0 represents no thrombosis; a score of 1, a few macroscopic strands of fibrin; a score of 2, several small thrombi; a score of 3, two or more large thrombi; and a score of 4 represents a single thrombus forming a cast of the lumen of the isolated segment

Normal Range of Values: Under the conditions of the assay as described above, normal human serum (1.32 ml./Kg.) will induce a score 4 thrombus in 96 per cent of test rabbits. There appears to be no important interassay variation and probit analysis of score 4 thrombi provides a practical method for the quantitative assay of the STA activity of human serum preparations Assays can also be evaluated from the percentage of animals that show any one of the previously defined scores They can thus yield quantitative data on serum preparations that induce only partial thrombosis in the doses given.

Precautions and Sources of Error: STA activity exhibits some thermolability and the duration of the hypercoagulability induced by its infusion is brief Therefore, it is recommended that the details concerning temperature, time of injection, and time of vein isolation be followed precisely Elaboration of STA activity will not occur if serum is collected, processed, and stored in siliconized equipment; it is, therefore, important that clean uncoated glassware be used throughout

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5. Flow Chamber Methods for the Quantitative Study of Thrombus Formation (Method of H. G. Downie, E. A. Murphy, J. F. Mustard and H. C. Rowsell[1])*

J. F. MUSTARD

Object: The object of the test is to provide a quantitative method for the study of thrombus formation in a high-flow pulsatile system in contrast to those methods employing stasis or vessel wall injury. The method may also be used for studying the morphology and histochemistry of thrombi formed in high-flow pulsatile systems.

Principle of the Test Rowntree and associates in 1927[1] and Best, Cowan and MacLean in 1938[2] developed an extracorporeal shunt technic to study the sequence of events in thrombus formation. We have adopted these technics in the study of the effects of such factors as flow on the pattern of thrombus formation and have subsequently found that such methods can be used for the quantitative measure of thrombus formation in a high-flow, pulsatile system. The extracorporeal shunt provides a constant surface and flow pattern The nature of the circulating blood can, therefore, be modified and its influence on thrombus formation examined

Reagents and Apparatus Required: A model of a vessel bifurcation has proved the most satisfactory. The models used are of two types. The first model is made from plastic with a symmetrical 60° bifurcation; it is made in two halves so that at the completion of the experiment it can be taken apart and the thrombus material formed in the lumen removed, transferred to a beaker, dried and weighed The second type of chamber which can be used is a disposable, plastic bifurcation model of smaller size Standard lengths of plastic tubing (Tygon, Nalgon) of the same bore as the flow chamber connect the flow chamber to intravenous cannulas which are inserted into the carotid artery and jugular vein. The streams from the two channels of the bifurcation are funnelled by a glass Y junction into a common tube ending in the jugular cannula The cannulas should not have a diameter less than half of that of the chamber A small piece of latex tubing (the "sampler") is introduced into both ends of the system to allow an area for sampling of blood, the administration of drugs, and the injection of air bubbles for the estimation of blood flow (fig 1) When the extracorporeal system is assembled the interior is coated with silicone (G E. dry film 87C) It has been found that it is necessary to use large

* Supported in part by a grant from the United States Publ[illegible]

domestic animals (weight greater than 18 Kg.) to obtain satisfactory results with the large flow chamber because so large a shunt would cause circulatory embarrassment in a smaller animal The smaller disposable chamber has, however, been successfully used in large rabbits.

Steps in the Performance of the Test: The animals are lightly anaesthetized and following anesthesia the carotid artery and jugular vein are

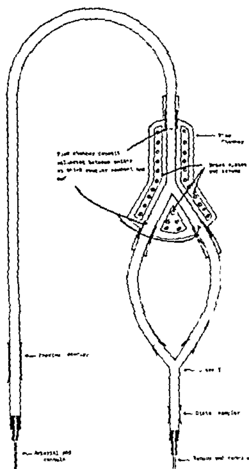


FIG 1 —Diagram of flow chamber and connections This illustrates the system used for the large non-disposable flow chambers The two halves of the chambers are held together by means of screws and nuts joining brass plates on the upper and lower surfaces

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Normal Range of Values: The normal range of values would have to be determined for each type of animal used and for each chamber. The deposit (for our chambers where the blood was allowed to flow for a 20-minute period) formed by 18 normal pigs had a geometric mean weight of thrombus of .377 mg. with 95 per cent confidence limits of .142-1.000 mg

Values are influenced by the nature of the circulating blood. For example, the geometric mean weight of thrombus formed in 22 swine receiving dicumarol, with prothrombin times between 12 and 18 seconds, was 4 89 mg —over 10 times as much as that formed in the controls. It has been found that high doses of dicumarol producing prothrombin times greater than 35 seconds lead to some diminution in the amount of thrombus material formed. From this and other studies, it can be shown that the values are influenced by altering the characteristics of the circulating blood Comparable observations have been made using the disposable plastic chamber in rabbits.

Precautions and Sources of Error: The entire experiment should be carried out at a constant temperature. The flow through the chamber should be constant over the period of time for the experiment and should be within pre-established limits

We have found that petroleum jelly should not be used to produce water-tight junctions as it may interfere with staining reactions, notably for fat.

In separating the two halves of the flow chamber, care must be taken not to dislodge the deposits as it may not be clear subsequently whether a piece of deposit formed within the lumen or from blood seeping between the connections We have found the best method to be to insinuate scissors between the two halves of the flow chamber and cut any strands bridging the gap between them.

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exposed with as little trauma as possible. The silicone-coated, polyethylene cannula filled with physiologic saline (0.85 per cent) are inserted one in each vessel. The entire extracorporeal circulation is filled with physiologic saline, and should be bubble-free. Hemostats closing the venous side of the chamber are removed and at a prescribed time the hemostat closing the carotid cannula is removed, and flow then commences. During preparation of the flow chamber, two marks are made in the proximal tube such that 20 ml. of saline fill the tubing between them. The time in seconds taken for the blood to flow from the one point to the other is noted In order to estimate blood flow at subsequent times, a 2 ml bubble large enough to extend across the entire lumen is introduced through the proximal latex tubing and the same measurements as before are made.

Flow continues for an exact specified period of time to enable comparisons to be made In our experience we have found 20 minutes to give satisfactory results After completion of the time of blood flow, the arterial hemostat is re-applied, the system is opened at the proximal end and two 100 cc. washings with physiologic saline are made to clear the chamber of blood The entire unit is then removed from the animal, and the chamber is dismantled and opened carefully. The deposits are then collected from the inner surface of the lumen in each half of the chamber This deposit is then placed in small pre-weighed beakers, dried and weighed

The disposable chambers are dried and weighed at the completion of the experiment The difference between the weight of these chambers before and after the experiment is taken as the weight of deposit formed

Manner of Expression of Results The amount of thrombus for both chamber systems is directly expressed as mg of dried deposit.

Calculations. Flow is calculated in ml of blood per minute This is based on the known volume of blood which is displaced per unit of time If the time taken for the bubble to move from one mark to the other be T seconds, then the volume flow in ml /minute (V) is given by $V = \frac{1200}{T}$ The calculated flow is adequate if it lies between 200 and 700 ml /minute This depends to some extent upon the depth of anaesthesia. We have found that, over this range, flow has little effect on weight of deposit ($r = -0.048$, $p < 0.5$)[3]

Examination of over 100 experiments using swine has shown that the weights of the deposits were lognormally distributed This is not surprising since, up to a point, it is to be expected that the rate at which a thrombus grows will be to some extent influenced by its size and the amount of coagulant active material liberated by it; that is, it will have a quasi-exponential growth.

Steps in the Performance of the Experiment: Human blood drawn in chilled siliconed equipment is mixed with one-tenth volume of I^{131} fibrinogen and the mixture is introduced into a straight glass tube to a volume of 8 ml. This volume contains between 50 and 70 μc. After incubation at 37°C. for at least 15 hours, to permit clot retraction, the clot is removed and washed with saline on a Buchner funnel. It is then stored overnight or for more than 1 day in cold phosphate buffer at 4°C. It is inserted as a pulmonary embolus in the dog via the inferior vena cava which is cannulated with a short piece of curved glass tubing connected to a glass cannula of 12 mm I.D. through which the radioactive clot is flushed into the circulation. The probe-type scintillation detector is secured over the point of maximum radioactivity over the thorax A 1-inch lead collimator is used on the probe. Urine is collected by a Foley catheter and divided into hourly fractions. Mannitol solution is delivered via a superior vena caval cannula by sigmamotor pump at the rate of 4 ml. per Kg. of body weight per hour. Blood samples are collected in one-tenth volume ammonium oxalate. Thrombolytic agents are administered by infusion into the femoral vein from a syringe injector with water jacket to maintain refrigeration of heat-labile enzymes

Calculations The experimental embolus is weighed before insertion and after recovery at necropsy Blood and urine samples are counted in a well and corrected for isotope decay Radioactivity excreted per hour is derived from total volume of urine flow multiplied by the specific radioactivity of the sample.

Alternate Procedures A second thrombolytic agent may be given following a period long enough to permit disappearance of the effect of the first agent from the circulation When an agent of unknown potency is under investigation, its administration may be followed at a later time by the infusion of a potent agent of known activity Should the first agent fail to induce lysis of the experimental embolus, the second agent, if effective, will demonstrate that the embolus is not insensitive and that its sensitivity is related to the potency of the agent [1]

Normal Range of Values Immediately after insertion of the embolus, there is a large output of radioactivity in the urine and high blood levels of radioactivity Both of these and the level of radioactivity observed over the thorax decline gradually, reaching a steady state at the end of the first 24 hours, which is roughly comparable among the experiments After this, administration of agents under study may begin Partial lysis may be noted as an unequivocal change in one or more of the following: decrease in chest radioactivity, increase in blood or urine radioactivity Complete lysis is indicated by marked changes in all of these values,

CHAPTER III

EXPERIMENTAL DESTRUCTION OF THROMBI

1. *Method for Determining Lysis of Experimentally Produced Thrombi**

M. HUME

Object of Experiment: Demonstration in vivo of clot lysis following administration of fibrinolytic enzymes

Principle Underlying Experiment. Whole blood, allowed to clot and retract, provides a substrate for the experimental demonstration of thrombolysis which is not unlike blood clots arising from thrombosis in major veins An experimental embolus made radioactive by combining I^{131}-labeled fibrinogen with whole blood can, after insertion into the circulation, be observed continuously by external monitoring over the point of maximum radioactivity Radioactivity observed externally and in serial samples of the blood and urine is related to changes in the weight of the embolus as measured at necropsy, and provides evidence of its stability in the circulation

Apparatus Probe-type scintillation detector with rate meter, read out on a chart-type recorder, sigmamotor infusion pump; fractional urine collector, syringe injector with ice-water jacket, well counter with pulse height analyzer adjusted for I^{131}, and appropriate shielding, standards and other materials for handling isotopes in intermediate amounts.

Reagents Anesthesia—Dial with urethane Anticoagulant for drawing blood samples—*0 1 M ammonium oxalate Mannitol infusion*—Mannitol USP 75 Gm , NaCl 18 Gm , dextrose 75 Gm , Lugol solution 1 ml , water 1500 ml The method of Eisen and Keston is used to label human fibrinogen (Cutter) †

* Investigations leading to this method were supported in part by research grants H-5140 from the U S P H S and 60 G 38 from the American Heart Association The author is a Markle Scholar in Academic Medicine

† The Eisen and Keston method is described on p 258

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2. Fibrinolytic, Caseinolytic and Biochemical Methods for the Study of Thrombolysis in Man: Application and Standardization*

A. J. JOHNSON, W. R. McCARTY, W. S. TILLET, A. O. TSE, L. SKOZA, J. NEWMAN and M. SEMAR

A. GENERAL CONSIDERATIONS

Various fibrinolytic agents are infused, to test their ability to lyse artificially induced blood clots, by initiating and maintaining a biochemically defined thrombolytic system.[1] As described in figure 1, these agents are: a kinase (streptokinase), an activator (urokinase), or plasmin (glycerol-activated). (1) Streptokinase (SK), or the kinases from tissue and plasma, appear to act stoichiometrically with plasminogen (proactivator)† to form an activator complex.[2,3] (2) This complex, like the naturally occurring activators in urine (urokinase), plasma or tissues, reacts catalytically with plasminogen (the proteolytic precursor)† to form a proteolytic enzyme, plasmin (fibrinolysin).[4] (3) Plasmin, in turn, acts upon fibrin, fibrinogen, and other native proteins, splitting them into soluble products.[5]

The intravenously infused fibrinolytic agent must be uninhibited or "free" in the circulating plasma to produce thrombolysis in man This can be achieved by neutralizing the total amount of circulating endogenous inhibitors (fig 1) with quantitatively equal amounts of the thrombolytic agent.[2] Since plasma contains endogenous inhibitors to each stage of the thrombolytic system, (1) SK may be neutralized by SK antibody and also by kinase inhibitor found in the alpha-2 globulin fraction of human sera;[6]

* Supported in part by U S P H S, National Institutes of Health Grant HE-0500-05, the Life Insurance Medical Research Fund G-63-20, and Lederle Laboratories of the American Cyanamid Co

† Plasminogen (proactivator) and plasminogen (proteolytic precursor) are separate functions of plasminogen which have not yet been identified as separate proteins

particularly in the radioactivity measured over the chest. Delay in the onset of lysis and duration of lysis can be read from the chart recording of externally observed radioactivity.

Precautions and Sources of Errors: The experimental embolus should be neither so stable as to be insensitive to the agents administered, nor so unstable as to disintegrate spontaneously during the period of study (48 hours) It is necessary to examine the clotted blood of several volunteers to select a donor whose blood clot remains maximally firm on retraction at 37°C. Although the experimental embolus is washed and stored in cold buffer to remove "loosely-bound" radioactivity, when it is introduced as an embolus some radioactivity appears promptly in the circulation and somewhat later in the urine It is apparent that the mechanical action of the circulation frees radioactivity from the experimental embolus during this period. Ordinarily, a stable state is reached during the first 24 hours, as recorded by a scintillation detector over the chest. Occasionally, a progressive decline towards background in this external radioactivity, still continuing at the end of 24 hours, is seen and is evidence that the embolus is not stable. Such a preparation is not used for evaluation of thrombolytic agents

The volume of diluent in which thrombolytic agent is administered may affect urine flow, particularly if the volume is large. Part of this may be a direct diuretic effect of the enzyme itself, in addition to the fluid load. Accordingly, it is necessary to correlate changes in the output of radioactivity in the urine with changes in the blood and over the chest. In addition, the specific radioactivity of the urine samples should increase if clot lysis is taking place

A steady level of anesthesia is obtained with a long-lasting anesthetic, Dial with urethane Too deep anesthesia results in deterioration of the animal preparation Too light anesthesia permits motion of the animal underneath the scintillation detector and introduces the artifact of sudden change (usually a decline) in the observed radioactivity over the chest. Intubation of the trachea with a cuffed tube is desirable to prevent aspiration of the gastric contents

Controls In each experiment, the period before administration of the thrombolytic agent serves as a control to the period of administration. Emboli weighed before insertion and after necropsy are compared with weight changes in similar emboli kept in buffer or plasma at 37°C Variation can be expected in the stability of emboli as determined by weighing, and this control is not so reliable as comparison of observed radioactivity before and after administration of thrombolytic agent. In addition, experiments are carried out in which no agent is given

warmed at 37.5°C. (7) 30 ml. syringe. (8) Meter stick (9) Two infusion sets.

Procedure A moderate-sized vein without branches (usually in forearm) is entered with a #18 needle for introduction of the dental broach (fig 2). With the blood pressure cuff adjusted to systolic pressure, the broach is completely turned clockwise, then counterclockwise several times and moved back and forth on its longitudinal axis for about 5 minutes. On removal of broach and needle, local hemostasis is achieved with digital pressure A sponge-rubber pad and Ace bandage are then applied proximal to the irritated area for 18-24 hours.

Venograms employing Hypaque are made with and without retrograde pressure of 40 mm Hg by means of a blood pressure cuff A standard injection pressure of 110 mm water may be obtained with gravity flow through an infusion set. Venograms are made (1) before clot induction,

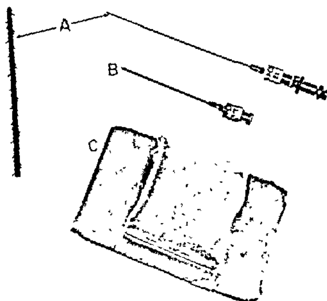


FIG 2.—Apparatus for induction of blood clots in peripheral veins (A) Dental broach soldered in tip of 2-inch, #21 needle (photomicrograph at left showing tip and barbed shaft) (B) Thin-wall #18 needle and stylet for venotomy to introduce dental broach (C) Sponge-rubber pad backed with stainless steel, for local pressure on proximal portion of vein

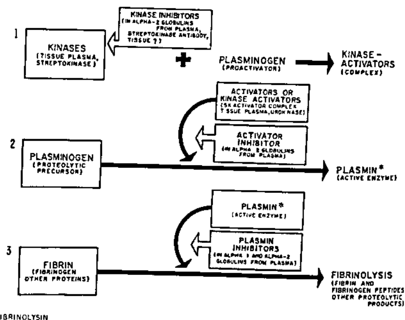


FIG 1—Mechanism of fibrinolysis in man.

(2) urokinase, or activator, may be neutralized by other inhibitors in this same fraction;[6] (3) plasmin (fibrinolysin) is probably neutralized by two additional inhibitors found primarily in the alpha-1 and alpha-2 globulin fractions.[7]

B. INDUCTION OF A STANDARD INTRAVENOUS CLOT IN MAN: TECHNIC OF X-RAY VENOGRAPHY

(A. J. Johnson and W. R. McCarty)

Object of the Method To induce clot formation in human volunteers.

Principle Underlying the Method Irritation of the intima of a superficial vein, followed by local pressure proximal to the irritated area, slows the blood flow and permits formation and fixation of a "white" thrombus.[1]

Reagents and Apparatus Required (1) Extra-coarse dental broach (#6, Chas B Schwed Co., New York) with the tip rounded and the base soldered to a 2-inch, #21 needle (2) 2 cc syringe (3) Thin-wall, #18 needle and stylet to introduce broach into vein. (4) Sponge-rubber pad with stainless-steel backing, 2 × 3 × 1 inch, curved to fit arm. (5) Ace-bandage. (6) Diatrizoate sodium (Hypaque sodium 50 per cent, Winthrop Laboratories, New York) diluted to 13 per cent in normal saline and

warmed at 37.5°C. (7) 30 ml. syringe. (8) Meter stick. (9) Two infusion sets.

Procedure: A moderate-sized vein without branches (usually in forearm) is entered with a #18 needle for introduction of the dental broach (fig. 2). With the blood pressure cuff adjusted to systolic pressure, the broach is completely turned clockwise, then counterclockwise several times and moved back and forth on its longitudinal axis for about 5 minutes. On removal of broach and needle, local hemostasis is achieved with digital pressure. A sponge-rubber pad and Ace bandage are then applied proximal to the irritated area for 18-24 hours.

Venograms employing Hypaque are made with and without retrograde pressure of 40 mm Hg by means of a blood pressure cuff. A standard injection pressure of 110 mm water may be obtained with gravity flow through an infusion set. Venograms are made (1) before clot induction,

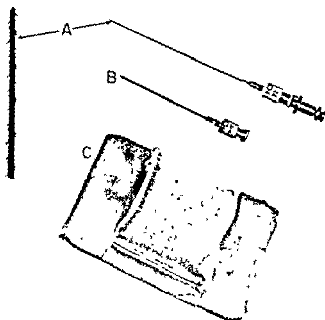


FIG 2.—Apparatus for induction of blood clots in peripheral veins. (A) Dental broach soldered in tip of 2-inch, #21 needle (photomicrograph at left showing tip and barbed shaft). (B) Thin-wall #18 needle and stylet for venotomy to introduce dental broach. (C) Sponge-rubber pad backed with stainless steel, for local pressure on proximal portion of vein.

(2) immediately prior to infusion of a thrombolytic agent, and (3) 24 hours after the infusion has been completed.

Precautions and Sources of Error: There must be no interruption in pressure (digital to sponge rubber) or the forming clot may fragment. The Ace bandage should be evenly applied without appreciably increasing the venous pressure. Otherwise edema of the entire distal arm will result. Pressure must be applied for 18-24 hours to fix the clot and prevent embolization. Diluting and warming Hypaque usually prevents further irritation and spasm of the already irritated vessel.

C. EXPERIMENTAL IN VIVO LYSIS OF CLOTS IN MAN BY AN ACTIVATOR SYSTEM

(A. J. Johnson, W. R. McCarty and J. Newman)

Object of the Method: To lyse blood clots in vivo by initiating and maintaining a carefully controlled, biochemically defined thrombolytic system in vivo, in the presence of widely variable activators and inhibitors in the circulating plasma [1,8]

Principle underlying the method: Control is established by determination of the amount, and kind, of inhibitors in vivo prior to infusion It is maintained during and after infusion by sequential determination of the amount, and kind, of fibrinolytic activity produced. In brief, a moderate activator system must be achieved in vivo without excessive plasminogen depletion and re-formation of the thrombus. This is accomplished by infusing SK at a rate which permits the formation of small amounts of plasmin, as well as moderate amounts of activator This method can also be used to lyse clots in vivo with urokinase

Reagents and Apparatus Required. Full listing appears under each test (1) Streptokinase (high purity for intravenous use, Lederle Laboratories, Division of American Cyanamid Co., Pearl River, N Y.). (2) 5 per cent dextrose in distilled water for intravenous use. (3) Normal serum albumin (human) salt-poor (collected and pooled by the American National Red Cross, Washington, D C, processed by E. R Squibb & Sons, N.Y.) (4) Epsilon aminocaproic acid (EACA), (Lederle Laboratories, Division of American Cyanamid Co., Pearl River, N Y.).

Procedure: The thrombolytic infusion may be divided into three progressive phases Phase I—neutralization of antibody and inhibitor (fig 3); Phase II—initiation of the fibrinolytic system (fig 3), and Phase III—maintenance of the fibrinolytic system (*Note* Each phase is carefully controlled by frequent testing. The relative importance of information contributed by each test is indicated by number of asterisks; *see foot-*

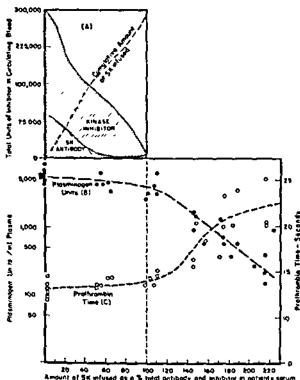


FIG 3—Effect in man, during phase I and phase II of intravenous infusion of SK, on Quick prothrombin time and blood levels of SK inhibitor, SK antibody and plasminogen (A) Decrease in circulating SK antibody and inhibitor due to cumulative infusion of SK (B) Decrease in circulating plasminogen, after neutralization of SK antibody and kinase inhibitor, indicating reaction with free SK (C) Increase in Quick prothrombin time, after neutralization of SK antibody and kinase inhibitor, indicating anti-thrombin effect of free plasmin (due to fibrinogen break-down products) *Note* Neutralization of both SK inhibitor and SK antibody (indicated by line at 100 per cent) is necessary to produce active fibrinolysis

notes. Only a few of these tests are critical: total SK inhibitor, whole-blood euglobulin clot lysis, Quick prothrombin time, plasminogen level)

In Phase I, total SK inhibition is determined by assaying the alpha-2 *inhibitor**** and *antibody**** in the patient's serum (fig 3). This value, in units of inhibitor per ml, when multiplied by the patient's estimated plasma volume (3000 ml for 70 Kg man) gives the number of SK units

*** Information from test is required

required for the initial or neutralizing dose (fig 3). The streptokinase is dissolved in a solution containing 5 per cent dextrose and 0 5 per cent human serum albumin. The full neutralizing dose of SK is infused at approximately 10,000 units/min.; the total dose may range from 100,000 to 2,000,000 units depending upon the inhibitor and antibody level in the serum. The presence of free plasmin, or activator, in the blood marks the end of Phase I as indicated by the *whole-blood euglobulin lysis test,**** *whole-blood clot lysis,*** *elevation in prothrombin time*** or *antithrombin*** (Seeger's method, see page 329), reduction in *fibrinogen,** and reduction to zero of *antibody** and *inhibitor** (fig. 3).

In Phase II, a thrombolytic system is initiated by continuing the infusion of SK after the neutralizing dose. The *plasminogen*** (proactivator method) drops sharply to 100-200 units/ml. of plasma (fig 3) Although 100,000 units of SK may be sufficient in Phase II for low inhibitor patients, the high inhibitor group may require 300,000-400,000 units The *fibrinogen** should be decreased at this time to about half its normal range and the *Quick prothrombin time**** increased to approximately 18 seconds (fig. 3). If SK is infused too slowly, too much *plasmin*** is formed, the *plasminogen*** level will exceed 300 units/ml of plasma, the *prothrombin time**** may be more than 18 seconds, and the *fibrinogen** level may fall below 100 mg per cent To correct this, SK must be infused at a faster rate. However, if SK is infused too rapidly, too much activator is formed, the *plasminogen level**** will drop below 100 units/ml of plasma, the *fibrinogen level*** will start to rise, the *Quick prothrombin time**** will drop below 18 seconds, and very little plasmin will be found in the plasma by the *casein method*** or *whole-blood euglobulin lysis time with 4 × 10^{-4}M EACA.***

In Phase III, the thrombolytic system is maintained by infusing SK at a relatively constant rate, usually 20,000-30,000 units/hr for low inhibitor patients, and 30,000-45,000 units/hr. for high inhibitor patients Again, too high an SK infusion rate is indicated by a *decrease* in (1) *plasminogen**** (below 100 units), (2) *Quick prothrombin time,**** (3) antithrombin VI,** and (4) *plasmin (casein method),*** and an *increase* in (5) *fibrinogen*** and (6) *activator* ** A small decrease in infusion rate up to 5000 units per hour usually corrects this Conversely, too slow an SK infusion rate is indicated by an *increase* in (1) *plasminogen**** (above 300 units), (2) *Quick prothrombin time**** (more than 25 seconds), (3)*antithrombin VI*** or (4) *plasmin (casein method),*** and a *decrease* in (5) *fibrinogen** and (6) *activator.** These changes from the steady state

* Information from test is helpful but not essential
** Information from test is required if not available by another method
*** Information from test is required

are sensitive indicators of the effect of the infusion rate. Obviously, in Phase III, the rate is very critical; free SK (or activator) must be provided, without excessive depletion of the endogenous plasminogen A patient may require two or three rate changes during one infusion In general, a steady state is initially maintained for 8-10 hours at a constant rate The plasminogen level then declines slowly and the infusion rate must be decreased by about 2000-5000 units per hour, 2 to 3 times per 24 hours. Since the duration of the infusion is directly proportional to the age of the thrombus, 48-hour-old thrombi should be infused for at least 48 hours and 24-hour-old thrombi for at least 24 hours. Even after thrombolysis has occurred, the infusion should be continued for a minimum of 4 more hours to permit partial intimal repair and prevent clot re-formation. Experimental thrombi 72 or more hours old require still longer periods of infusion; lysis is exceedingly difficult and sometimes impossible

D. ASSAY METHODS—FIBRINOLYSIS PRECURSORS AND INHIBITORS

1. General Principles Underlying the Methods (Fig. 1 and Table 1) (A. J. Johnson)

The final common pathway for each assay is the proteolytic degradation of fixed amounts of an *indicator substrate* (fibrin in a standard clot, or casein) by varying amounts of the *active enzyme* plasmin Thus, plasmin may be present as a preformed substance which may be assayed in a single-stage reaction by varying the tube dilutions of plasmin It may also be formed in a two-stage reaction by the interaction of variable amounts of *activator* (UK, or SK plus human plasminogen "proactivator") with a fixed quantity of *enzyme substrate* (human plasminogen "proteolytic precursor," or bovine plasminogen in excess) Assays utilizing the two-stage reaction may be subdivided into two categories (1) those assays utilizing limited activation of human plasminogen, where activator and plasmin form during the period of *incubation*, even as clot lysis occurs, and (2) those which require maximal activation of human plasminogen, where activator and plasmin are formed over a 20-minute period in a separate *pre-incubation mixture* The plasmin from this mixture is then added to an *indicator substrate* (fibrin or casein) during the period of incubation *(incubation mixture)*

Inhibitor assays, on the other hand, are performed by first neutralizing fixed quantities of plasmin, SK, or UK, with varying dilutions of inhibitor in a separate *pre-incubation mixture* Residual free *plasmin* (in the case of plasmin inhibitor) acts upon an *indicator substrate*, residual *activator* (in

TABLE 1. *Principles of Fibrinolytic and Caseinolytic Methods for the Study of Thrombosis*

FUNCTION MEASURED	PRE-INCUBATION MIXTURE		INCUBATION MIXTURE 37.5°C		OPTIMAL RANGE	UNIT	CONTROLS	NORMAL RANGE† IN CIRCULATING BLOOD (MAN)	SECTION
	Neutralization	Activator + Substrate	Activator + Substrate	Active Enzyme + Indicator Substrate					
Plasmin Activity	⟶	⟶	⟶	Plasmin + Casein or Fibrin Clot†	0.1 – 0.600 6-20 min lysis	0.0275 Tr / 1 µg/min 10 min lysis	Standard Plasmin	Negligible	0-9 0-8
UK (Activator)			UK† + HP†	Plasmin and UK + Fibrin Clot†	6-20 min lysis	10 min lysis			0-10a
UK (Activator)		UK + HP 20' at 37.5°C		Plasmin and UK + Casein or Fibrin Clot†	0.1 – 0.600 4-40 min lysis	0.0275 Tr / 1 µg/min 10 min lysis	Standard UK	Negligible	0-11 0-10b
SK (Activator)	⟶	⟶	SK† + HP†	Plasmin and SK-activator + Fibrin Clot†	6-20 min lysis	10 min lysis	Standard SK	None	0-10a
SK (Activator)	⟶	SK + HP 20' at 37.5°C	⟶	Plasmin and SK-Activator + Casein or Fibrin Clot†	0.1 – 0.600 4-40 min lysis	0.0275 Tr / 1 µg/min 10 min lysis			0-11 0-10b
Plasmin Inhibitor	1 1/2 un CP + inhibitor (naturally occurring or synthetic) 10 min room temp	⟶	⟶	Residual Plasmin + Fibrin clot†	6-20 min lysis	10 min lysis	1 1/2 & 1 unit plasmin	150-250 units per ml serum or plasma	0-15
UK Inhibitor	1 unit UK + inhibitor (serum plasma or synthetic) 10 min 4°C	⟶	Residual UK† + HP†	Plasmin and UK + Fibrin Clot†	10-25 min lysis	10 min lysis	1 unit & 1/2 unit UK	35-90 units per ml serum or plasma	0-14
SK Inhibitor + SK Antibody	1 unit SK + serum other biolog fluid (no chelating agent) 10 min 4°C	⟶	Residual SK† + HP†	Plasmin and SK activator + Fibrin Clot†	10-25 min lysis	10 min lysis	1 unit & 1/2 unit SK	80-120 units per ml serum	0-14

Plasminogen Content (Proactivator)	→	→	1000 u [illegible]† + [illegible] and [illegible]†	Plasmin (Human and Bovine) and SK activator + Fibrin Clot‡	≥ 20 min lysis	10 min lysis	Standard Human Plasminogen	3000-10 000 units/ml Plasma	D-13
Plasminogen Content (Proteolytic Precursor)	→	50 units SK + 50 5000 u HP (20' at 37.5°C)	→	Plasmin and SK-activator + Casein or Fibrin Clot‡	0.1 - [illegible] 6-40 min lysis	0.0 [illegible] 1 µg/min 10 min lysis	Standard Human Plasminogen	[illegible] ml Plasma 130 u/ml Plasma	D-13 D-13
Bovine Plasminogen	→	1000 u SK and 70 u HP or 50 u UK + BP (20' at 37.5°C)	→	Plasmin and SK activator or Plasmin and UK + Casein or Fibrin Clot‡	0.1 - [illegible] 6-40 min lysis	0.0 [illegible] 1 µg/min 10 min lysis	Standard Bovine Plasminogen	None	
Activator and Plasmin in Whole Blood Euglobulin	→	→	Endogenous Activator Plasminogen and Plasmin	Fibrin Clot†	None	None	None	Clot lysis in 1 1/2 hr without added EACA and in 3 1/2 hr with added EACA	D-7

‡ FIBRINOLYTIC UNITS UNLESS REFERRED TO AS CASEIN UNITS

† REAGENTS ADDED IN COLD. 37.5°C INCUBATION AFTER CLOT IS FORMED

‡ REAGENTS ADDED PRIOR TO CLOT FORMATION

B P - PARTIALLY PURIFIED BOVINE PLASMINOGEN ([illegible] FIBRINOLYTIC UNITS PER [illegible]) CONTAINED IN PARTIALLY PURIFIED BOVINE FIBRINOGEN (FI)

CAS PURIFIED ALPHA CASEIN VERY KINDLY SUPPLIED BY DR. [illegible] UNITED STATES DEPARTMENT OF AGRICULTURE AGRICULTURAL RESEARCH SERVICE,
STANDARD REFERENCE LOT NO. [illegible]

FI PARTIALLY PURIFIED BOVINE FIBRINOGEN ARMOUR PHARMACEUTICAL CO. STANDARD REFERENCE LOT NO. [illegible] AND RABBIT THROMBIN
LEDERLE LABORATORIES DIVISION AMERICAN CYANAMID CO. STANDARD REFERENCE LOT NO. 308

PL - GLYCEROL ACTIVATED PURIFIED HUMAN PLASMIN AMERICAN NATIONAL RED CROSS STANDARD REFERENCE LOT MICHIGAN LOT NO. 6

UK UROKINASE ABBOTT LABORATORIES REFERENCE LOT NO. [illegible] STERLING WINTHROP RESEARCH INSTITUTE STANDARD REFERENCE LOT NO. [illegible]

HP PARTIALLY PURIFIED HUMAN PLASMINOGEN AMERICAN NATIONAL RED CROSS STANDARD REFERENCE LOT NO. [illegible]

SK STREPTOKINASE LEDERLE LABORATORIES DIVISION OF AMERICAN CYANAMID CO. STANDARD REFERENCE LOT NO. [illegible]

TY TRYPSINE

the case of SK and UK inhibitors) is first reacted with *enzyme substrate* to form plasmin, which then acts upon an *indicator substrate.*

2. General Precautions and Sources of Error for Methods (A. J. Johnson)

(1) Accidental hemolysis in the sample may result in erroneously decreased inhibitor and increased plasmin and activator. (2) Samples of biological fluids should be adjusted to a pH range of 7.0-8.0 before assay (3) Use of gelatin buffer in assays with low concentrations of SK or UK prevents adsorption of the enzymes by the glassware. (4) Log dilutions of a sample may be rapidly and accurately made by the micrometric syringe buret The following technics are essential to quantitative accuracy in use of the buret: (a) the assay tube must contain 0.1 ml. of buffer (or equivalent) during delivery from the syringe; (b) the syringe tip must be below the fluid level; (c) the tip must be carefully wiped with tissue (e g , Cellu-wipes) before delivery to each tube, and (d) the buret should be well-rinsed and autoclaved at 15 psi for 15 minutes between assays to denature any residual reagent (5) Required reagents should be added to a series of tube dilutions (for a particular assay) in rapid sequence and in order of increasing concentration of. (a) reagent added, or (b) reagents already present in the tubes. (6) When all reagents have been added, the tubes must be promptly agitated to ensure mixing before the clot forms (7) After lysis starts, the tubes should be observed at intervals of less than 30 seconds by removing them from the water bath for only a few seconds. (8) Excessive agitation of the tubes after the clot has formed causes aggregation of the fibrin and slows the rate of lysis.

3. Estimation of Fibrinolytic End Point in Purified Systems

Although plasmin has little proteolytic activity against many physiologic substrates, it can depolymerize the fibrin in a standard fibrin clot (fibrinolysis) at a rapid rate. The rigid, opalescent, fibrin gel gradually changes to a water-clear, free-flowing solution of fibrin split products There are at least two principal methods of gauging plasmin's effect on the indicator substrate fibrin (1) gentle tilting of the tube to about 45° which aids visualization of the decrease in clot viscosity; and (2) observation of bubbles formed during incubation of the clot at 37 5°C. which remain trapped until a marked decrease in clot viscosity permits them to float to the surface A precise end point can be obtained more consistently in a wide variety of assays when performed by the tilt-tube method, but it is also a more difficult end point to identify The average technician can readily perform SK assays with partially purified reagents using the bubble method, but will find that it is more difficult to obtain a precise end point in other

assays with highly purified reagents. Assay results with the bubble end points tend to be approximately 15 per cent higher than those obtained with the tilt-tube method.

Utilizing fibrin as the indicator substrate in the system of fibrinolytic assays presented below for control of thrombolysis, the fibrinolytic units of SK, UK, plasminogen and plasmin, as well as those of inhibitors of SK, UK and plasmin, have been based upon the enzyme activity required to lyse a standard clot in 10 minutes at 37.5°C. Thus, the units of fibrinolytic activators and inhibitors are interconvertible.

4. *Preparation of Buffers and Reagents for Fibrinolytic and Caseinolytic Assays*

BUFFER TABLE

Saline (0 07M)-Phosphate (0 06M) Buffer pH 7.5

NaCl	4 092 Gm.
Na_2HPO_4	7.156 Gm.
KH_2PO_4	1.306 Gm.
Merthiolate*	10 mg

Dissolve in approximately 900 ml distilled water. Adjust to pH 7.5 and bring to final volume of 1 liter.

Gelatin Saline-Phosphate Buffer pH 7.5

Purified calfskin gelatin 5 0 Gm.
Reagents for saline-phosphate buffer
Dissolve gelatin in 500 ml. distilled water with gentle heating and stirring Add saline phosphate buffer reagents and add distilled water to approximately 900 ml Adjust to pH 7.5 and bring to a final volume of 1 liter

Tris (0 06 M)-Saline (0 09 M) Buffer pH 7.5†

Tris	7.265 Gm
Tris hydroxymethyl aminomethane	
NaCl	5 26 Gm.
Merthiolate (Eli Lilly Co) 10 mg *	

Dissolve in approximately 900 ml distilled water. Adjust to pH 7 5 (with concentrated HCl) and bring to final volume of 1 liter.

* No merthiolate is used in buffer when used for casein assay because of high absorbance at 275 mμ When used without merthiolate, buffer should be tubed in aliquots and stored in deep freeze until day of use

† Buffer frozen in small aliquots is stable for 2 weeks Thawed buffer should not be refrozen Discard at end of day

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Dissolve in distilled water. Add 2×10^{6} units of Kunitz pancreatic trypsin inhibitor (Iniprol)* or 10,000 units of Trasylol.† Adjust to pH 6.4 (HCl) and dilute to total of 1 liter.

Reagent Table

Fibrinogen: Five and six-tenths mg. aliquots of bovine fibrinogen, as clottable protein (or 12.5 mg. of standard lot no. 6211, Armour Pharmaceutical Co., Kankakee, Ill.), are weighed and stored in test tubes in several vacuum desiccators with anhydrous phosphorous pentoxide as the desiccant. The desiccators are used in rotation to keep the fibrinogen as dry as possible. Immediately before use, 0.5 to 1.0 ml. of buffer‡ is added to each tube, and a glass rod is pressed against the tube sides to wet the fibrinogen; more buffer‡ is added to bring the volume to 2.5 ml. The solution is stored on ice until used.

Plasminogen: Partially purified human plasminogen, Cohn fraction III (American National Red Cross) is weighed in 15,000-unit§ amounts and stored in test tubes, until needed. Prior to use, 5 ml. of buffer‡ is added; the mixture is stirred vigorously against the sides of the tube for 5 minutes with a glass rod and diluted to 50 ml.-final volume. The tube is then kept on ice until used. Undissolved fraction III does not seem to interfere with the assay; thus the suspension may be centrifuged and the clear supernatant solution used for assay, although this step is not necessary.

Thrombin: A stock solution of rabbit thrombin (Lederle Laboratories, Division of American Cyanamid Co., Pearl River, N.Y.) made by adding 80 N.I.H. units/ml. to 50 per cent glycerol in acetate buffer, pH 5.8 (1 Gm. of thrombin Lot #308 + 12.5 ml. glycerol and 12.5 ml. of acetate buffer) can be kept on hand under refrigeration for long-time use. A working solution of 20 N.I.H. units/ml. is prepared by diluting the stock solution with buffer.‡ It is kept on ice until needed.

Streptokinase: SK (Lederle Laboratories) is diluted in gelatin buffer‖ (10,000 units/ml.). Aliquots frozen immediately at −20°C remain stable for 3-6 months. The frozen mixture is thawed and diluted in gelatin buffer‡ prior to use to 10 units/ml. for SK inhibitor and SK antibody assays. The 10,000 units/ml. SK is used without further dilution for fi-

* Laboratoire Choay, Paris, France.
† FBA Pharmaceuticals, Inc., New York, N.Y.
‖ Either gelatin S.P. or gelatin Tris-saline buffer, depending on the buffer system specified in assay.
‡ Either S.P. or Tris-saline buffer, depending on which buffer is specified in the assay method.
§ 50 mg. crude Fraction III, Lot #1897.

*Gelatin Tris-Saline Buffer pH 7.5**

Purified calfskin gelatin (Eastman Kodak Co.)	5.0 Gm.
Reagents for Tris-saline buffer	

Dissolve gelatin in 500 ml. distilled water with gentle heating and stirring. Add Tris-saline buffer reagents and distilled water to approximately 900 ml. Adjust to pH 7.5 (with concentrated HCl), and bring to final volume of 1 liter.

0.073 M Acetate Buffer pH 5.8 in 0.070 M Saline

Sodium Acetate	6.0 Gm.
NaCl	4.09 Gm.
Merthiolate	10 mg.

Dissolve in distilled water. Adjust pH to 5.8 (glacial acetic acid) and bring to final volume of 1 liter.

Buffer for Preparation of Fibrinogen Deficient in Plasminogen

0.075 M Phosphate Buffer pH 6.4

KH_2PO_4	7.35 Gm.
Na_2HPO_4	2.98 Gm

Dissolve in distilled water. Adjust to pH 6 4 and dilute to a total of 1 liter.

Buffers for Fibrinogen Determination

0 015 M Phosphate Buffer in 0 075 M NaCl and 1 M EACA pH 6.35

KH_2PO_4	1 63 Gm.
Na_2HPO_4	0 426 Gm
NaCl	4 39 Gm.

EACA (epsilon amino caproic acid) 131 1 Gm

Dissolve in distilled water Adjust to pH 6 35 (HCl) and dilute to a total of 1 liter.

0 02 M Imidazole Trypsin Inhibitor Buffer with 0 05 M Calcium Chloride in 0 08 M Saline pH 6 4

Imidazole	1 36 Gm
$CaCl_2$	5 55 Gm
NaCl	4 68 Gm

* Buffer frozen in small aliquots is stable for 2 weeks Thawed buffer should not be refrozen. Discard at end of day.

(2) 2 N HCl (3) 0.075 M phosphate buffer, pH 6 4 (see Buffer Table, page 459). (4) EACA. (5) Glycine. (6) Saturated $(NH_4)_2SO_4$. (7) 0 3 M KCl in 0.4 per cent trisodium citrate. (8) 2 N NaOH. (9) Whatman #12 filter paper, 32.0 cm diameter. (10) 180 mm diameter funnel. (11) Glass stirring rod. (12) 3-liter Erlenmeyer flask. (13) 500 and 1000 ml graduated cylinders (14) Refrigerated centrifuge. (15) Refrigerator. (16) Distilled water. (17) Phosphate EACA buffer or Imidazole trypsin inhibitor buffer pH 6 4 (see Buffer Table, page 460). (18) Topical thrombin (Parke, Davis & Co.) 500 N.I.H. units/ml. normal saline. (19) Flat polyethylene tubing, 7/8 inch wide, 0 002-inch single thickness (Chippewa Plastics Co, Division of Rexall Drug and Chemical Co, Chippewa Falls, Wis.), made into 6-inch heat-sealed bags. (20) Normal saline. (21) Spectrophotometer. (22) Crystalline bovine albumin (Armour Pharmaceutical Co., Kankakee, Ill). (23) 25 per cent normal serum albumin (human), salt-poor (collected and pooled by the American National Red Cross, Washington, D C., processed by E R. Squibb & Sons, New York), Kjeldahl-standardized (24) 5/8 × 4¼ inch Lusteroid tubes (Lusteroid Container Corp., Maplewood, N.J)

Procedure for Purification: (a) Four Gm of fibrinogen are dissolved in 200 ml. of 0 075 M phosphate buffer, pH 6.4, by stirring gently with a glass rod. Then (b) 200 ml. of distilled water is added and enough 2 N HCl (about 5 drops) to bring the pH to 6 4. (c) After the solution has been refrigerated overnight, it is decanted and filtered without disturbing the precipitate which is discarded (d) EACA and glycine (both in powdered form) are added to the water-clear filtrate in amounts to bring the final concentration to 0 2 M for glycine and 0 1 M EACA (e) One hundred and thirty ml. of saturated $(NH_4)_2SO_4$ is slowly added with constant stirring, in the cold, and allowed to stand for at least 2 hours (f) The precipitate is recovered by centrifugation and dissolved in 60 ml of 0.3 M KCl in 0 4 per cent trisodium citrate (g) The pH is raised to 7 2 by adding a few drops of 2 N NaOH (h) The fibrinogen solution is dialyzed in the cold for 3 days against several changes of 0 3 M KCl in 0 4 per cent citrate. Steps (d), (e), (f), (g) and (h) are repeated 1 to 5 times, until 24-hour clot stability is obtained, even though 1-10 Christensen units of SK and 59-590 C T A units of UK are added

Procedure for Determining Clottable Protein The total protein content of Cohn fraction I or of purified fibrinogen solution is determined by the biuret method The clottable protein in either material is measured as follows 1 0 ml fibrinogen solution is placed in a polyethylene bag and 2 ml. imidazole or "fibrinogen" phosphate buffer is added The fibrinogen is clotted with 25 N I H. units of thrombin The bag is kept on ice for at least 1 hour, put in a Lusteroid tube containing 12 ml. of water as a

brinolytic plasminogen (proactivator) assays. The diluted SK remains stable at 4°C. for 8 hours.

Urokinase: UK (Abbott Laboratories and Sterling Winthrop Research Inc., Division of Sterling Drug Co) is prepared by the same method as SK Diluted UK (10 units/ml.) is stable for 1 week when frozen at −20°C. The more concentrated the solution, the longer it will remain stable.

Glycerol-activated Human Plasmin: Glycerol-activated human plasmin (prepared for the American National Red Cross by the Michigan State Department of Health) is stable for long periods of time under refrigeration. Immediately prior to each assay the plasmin is diluted to 10 units/ml with 25 per cent glycerol in buffer* and kept on ice

UK-activated Human Plasmin: Fifteen units of UK are added to 20 Gm. Cohn fraction III (American Red Cross), and carefully dissolved in 200 ml of S P. buffer The mixture is pre-incubated for 30 minutes at 37 5°C., with frequent stirring, and spun for 30 minutes at 27,000 g. in a refrigerated angle centrifuge to remove undissolved material The supernatant, kept at room temperature, is repeatedly assayed for plasmin activity until a plateau is reached (usually 3-5 hours). At this time, glycerol is added to a final volume of 50 per cent, to stabilize the plasmin The preparation is kept at room temperature Its activity gradually decreases for about 2 months, as determined by weekly assays, until identical assay results have been obtained for 4 consecutive weeks. The plasmin is then stored at 4°C, and retains full activity for more than 1 year

5. Preparation of Fibrinogen Deficient in Plasminogen, and Determination of Clottable Protein

(A. J. Johnson, A. O. Tse and J. Newman)

Object of the Method To prepare a plasminogen-deficient fibrinogen for use in fibrinolytic assays.

Principle Underlying the Method At pH 6 4 and low ionic strength, plasminogen and cryoglobulins precipitate while most of the fibrinogen remains in solution[9] The fibrinogen is then repeatedly reprecipitated with ammonium sulfate in the presence of 0 1 M epsilon aminocaproic acid (EACA) and 0 2 M glycine to solubilize the plasminogen,[10] which tends to remain in solution with each succeeding precipitation until there is little or none in the precipitate The fibrinogen is dialyzed for 3 days after *each* precipitation to free it of ammonium sulfate

Reagents and Apparatus (1) Low-plasmin Cohn fraction I, of bovine origin (Armour Pharmaceutical Co , Kankakee, Ill) or human origin (American National Red Cross), stable in standard clot for 24 hours.

* Either S P or Tris-saline buffer, depending on which buffer is specified in assay method

(b) 1.8 ml. of freshly drawn venous blood is immediately added to 0.2 ml. of 4×10^{-3} M EACA in one tube and 2 ml of blood is placed in the other. (c) Both tubes are closed with rubber stoppers from which runs a length of nichrome wire ending in a spiral below the 2.0-ml. level. (d) The tubes are gently mixed by inversion and promptly incubated at 37.5°C. They are observed periodically for lysis during the next 24 hours (e) The clot residue is then carefully removed after draining it against the side of the tube for 1-3 minutes, and the tubes are centrifuged at 4000-10,000 r p m for 15 minutes to pack cells freed by lysis, clot retraction, or both. (f) The total volume of serum and cells as well as packed cells is measured.

Calculations: The volume of packed cells in the tube is divided by 2 (for the initial clot volume of 2 0 ml) and compared to the volume of packed cells in the hematocrit (as 100 per cent)

Interpretation of Results: The normal range of red-cell "fall-out" in either tube is less than 20 per cent in 24 hours. When lysis occurs to the same extent in both tubes, it is due to plasmin. When it occurs only in the tube without EACA, it is due to activator. When there is partial lysis, due to both activator and plasmin, it will be more extensive in the tube without EACA.

Precautions and Sources of Error: (1) When fibrinolytic agents are infused to induce a thrombolytic system in vivo, a marked decrease in plasma plasminogen will prevent lysis of whole blood clots even in the presence of an excessive amount of activator. (2) In drawing the blood sample, the tourniquet or blood pressure cuff should not be used for more than 3 minutes since prolonged anoxia induces local activation of fibrinolysis. (3) A low fibrinogen level or polycythemia vera may be mistaken for fibrinolysis because the cellular residue will be larger than normal

7. Estimation of Fibrinolytic Activity by Whole-Blood Euglobulin Clot Lysis (A. J. Johnson, M. Semar and J. Newman)

Object of the Method. To assay fibrinolytic activity in circulating blood by in vitro measurement of fibrinolytic activity of the euglobulin fraction.

Principle Underlying the Method: The euglobulin fraction is precipitated from whole blood or plasma when the ionic strength is sharply decreased (with dilute acid) to about 0 008 at pH 5 2-5 9 [13,14] The precipitate contains activator and/or plasmin from the plasma and red cell stroma, as well as plasminogen and fibrinogen; but the major fibrinolytic inhibitors are either labile at this pH or remain in the supernatant. Thus, euglobulin fractionation tends to inactivate the endogenous inhibitors and separate them from the endogenous enzymes [14] Activator and plasmin are qualitatively differentiated when the precipitate is reconstituted by the addition of

cushion, the open top folded over the rim of the tube and fastened with a rubber band and centrifuged in the cold, at 15,000 rpm for 20 minutes. The bag is carefully removed from the tube and squeezed gently with the fingers to force fluid remaining in the clot out through the open top of the bag. The clot, still in the bag, is given three separate washes with ice-cold normal saline, and squeezed gently after each; this procedure is then repeated twice more with ice-cold distilled water. One ml of 1 N NaOH is added to the plastic bag to dissolve the clot (about 1 hour at 37.5°C.). Four ml. of biuret reagent[11] is added, and the mixture is reacted with the protein for 30 minutes at room temperature, the optimal protein content is 1-10 mg./ml. The protein-biuret solution is read against a blank of 4.0 ml. biuret reagent + 1 ml. of 1 N NaOH at 550 mμ in a spectrophotometer.

Calculation. The optical density is converted to mg. of protein on a standard curve calibrated with (1) Kjeldahl-standardized cryst. bovine albumin and (2) 25 per cent salt-poor human albumin. Reproducibility is ±0.5 per cent.

Precautions: Correction must be made for released fibrinopeptides since the biuret reagent measures these as well as the clottable protein. (b) The sample must be kept on ice in 1 M EACA, or other inhibitor (Reagent Table) to inhibit plasmin and prevent fibrin degradation from the moment it is taken (including the 1-hour clotting time). Blank for total protein is same buffer as in sample.

6. Estimation of Fibrinolytic Activity by Whole-Blood Clot Lysis (A. J. Johnson and W. S. Tillet)

Object of the Method: To determine fibrinolytic activity in the circulating blood, due to activator and/or plasmin.

Principle Underlying the Method. In the presence of excess substrate (plasminogen), spontaneous or induced fibrinolytic activity in vivo will lyse a whole blood clot in vitro if the amount of kinase, activator, and plasmin exceeds the amount of kinase, activator, and plasmin inhibitors.[1] The types of activity can be differentiated by adding EACA (an activator inhibitor) to the blood in vitro Fibrinolytic activity which is not inhibited by 4×10^{-4} M EACA is considered plasmin.[6,12]

Reagents and Apparatus. (1) Ten-ml hypodermic syringe. (2) #20 hypodermic needle. (3) 15-ml. graduated conical centrifuge tubes (4) Silicone (Beckman Desicote or equivalent) (5) Normal saline. (6) 4×10^{-3} M EACA. (7) #18 nichrome wire. (8) Size-1 rubber stoppers (9) 37.5°C. water bath (10) Clinical centrifuge.

Procedure. (a) Two centrifuge tubes are filled with silicone solution, drained well, air-dried, and rinsed several times with normal saline

(b) 1.8 ml. of freshly drawn venous blood is immediately added to 0 2 ml. of 4×10^{-3} M EACA in one tube and 2 ml of blood is placed in the other. (c) Both tubes are closed with rubber stoppers from which runs a length of nichrome wire ending in a spiral below the 2.0-ml. level. (d) The tubes are gently mixed by inversion and promptly incubated at 37.5°C. They are observed periodically for lysis during the next 24 hours. (e) The clot residue is then carefully removed after draining it against the side of the tube for 1-3 minutes, and the tubes are centrifuged at 4000-10,000 r p m for 15 minutes to pack cells freed by lysis, clot retraction, or both. (f) The total volume of serum and cells as well as packed cells is measured.

Calculations: The volume of packed cells in the tube is divided by 2 (for the initial clot volume of 2.0 ml.) and compared to the volume of packed cells in the hematocrit (as 100 per cent).

Interpretation of Results: The normal range of red-cell "fall-out" in either tube is less than 20 per cent in 24 hours. When lysis occurs to the same extent in both tubes, it is due to plasmin. When it occurs only in the tube without EACA, it is due to activator When there is partial lysis, due to both activator and plasmin, it will be more extensive in the tube without EACA

Precautions and Sources of Error. (1) When fibrinolytic agents are infused to induce a thrombolytic system in vivo, a marked decrease in plasma plasminogen will prevent lysis of whole blood clots even in the presence of an excessive amount of activator. (2) In drawing the blood sample, the tourniquet or blood pressure cuff should not be used for more than 3 minutes since prolonged anoxia induces local activation of fibrinolysis. (3) A low fibrinogen level or polycythemia vera may be mistaken for fibrinolysis because the cellular residue will be larger than normal

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Calculation: The optical density is converted to mg of protein on a standard curve calibrated with (1) Kjeldahl-standardized cryst. bovine albumin and (2) 25 per cent salt-poor human albumin. Reproducibility is ±0.5 per cent.

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Reagents and Apparatus: (1) Ten-ml. hypodermic syringe. (2) #20 hypodermic needle. (3) 15-ml. graduated conical centrifuge tubes. (4) Silicone (Beckman Desicote or equivalent) (5) Normal saline. (6) 4×10^{-3} M EACA (7) #18 nichrome wire. (8) Size-1 rubber stoppers. (9) 37.5°C. water bath. (10) Clinical centrifuge.

Procedure: (a) Two centrifuge tubes are filled with silicone solution, drained well, air-dried, and rinsed several times with normal saline.

the fibrinogen content of the clot. Therefore, it is necessary to know the approximate fibrinogen content in order to interpret the results accurately. (3) Low fibrinogen samples are characterized by a small precipitate, inadequate co-precipitation of activator and plasmin, and poor clot formation.

8. *Fibrinolytic Assay for Plasmin, Purified or in Biological Fluids* (A. J. Johnson and A. O. Tse)

Object of Method. To determine the amount of plasmin in purified systems and biological fluids.

Principle underlying the Method· When plasmin is mixed in the cold with fibrinogen and thrombin and incubated at 37.5°C., the resulting clot lysis time is inversely proportional to the amount of plasmin that is present (under standard conditions of time, temperature, pH, ionic strength, type of buffer, etc.[15,16]). The amount of plasmin which is required to lyse a standard clot in 10 minutes has been designated as 1 unit.

Reagents and Apparatus: (1) Sample containing plasmin (2) Bovine fibrinogen dissolved in S P. buffer, Reagent Table, page 461. (3) Working solution rabbit thrombin, Reagent Table, page 461. (4) S P buffer (5) 25 per cent glycerol in S P. buffer. (6) Micrometric syringe buret (Model SB2, Micrometric Instrument Co , Cleveland, Ohio) (7) 0 2, 1.0, 2 0, 5 0, and 10 0-ml. serologic pipettes. (8) 12 × 75-mm test tubes (9) Ice bath (10) 37.5°C water bath. (11) Stop watch.

Procedure: (a) Test tubes containing up to 0 7 ml. S P. buffer are placed in an ice bath and (b) serial 0 05 log dilutions of plasmin with 25 per cent glycerol in S P. buffer are added with micrometric syringe buret. (c) Two-tenths ml. fibrinogen, and (d) 0.1 ml thrombin are added to make final clot volume of 1 0 ml The tubes are agitated at once and placed in a water bath, where they clot, and the stop watch is started

Calculations. Clot lysis times are plotted against the reciprocals of the final dilutions of plasmin on log-log paper (fig 4) (A French curve may be used, if necessary, to construct curve of best fit) Alternatively, a straight line graph may be obtained by plotting the reciprocal of the lysis time against the reciprocal of the final dilutions of the sample on a semi-log plot The dilution lysing the clot in 10 minutes (as indicated by the 10-minute intercept on the curve) is designated as 1 unit of plasmin The reciprocal of this dilution equals the number of units/ml in the sample being tested

Precautions and Sources of Error In fibrinolytic assays for plasmin, small amounts of human or bovine plasminogen in the fibrinogen or thrombin will not give erroneous results if test sample contains no activator.

Christensen Fibrinolytic Units/ml
Standard Plasmin Lot #8

an activator inhibitor (4×10^{-4} M EACA) to one-half the tubes containing the euglobulin precipitate.[6,12] The reconstituted precipitate is then clotted with thrombin, incubated at 37.5°C., and observed for lysis.

Reagents and Apparatus Required: (1) Ten-ml. hypodermic syringe. (2) #19 hypodermic needle. (3) ⅝ × 4¼-inch Lusteroid tubes (Lusteroid Container Corp., Maplewood, N.J.). (4) 3.8 per cent trisodium citrate. (5) S P. buffer. (6) CO_2 gas or 1.0 per cent acetic acid (7) 2.8×10^{-3} M EACA. (8) Tropical thrombin (Parke, Davis & Co.) 40 N.I.H. units/ml. normal saline. (9) 12 × 75-mm. test tubes. (10) 0 2, 1.0, and 10 0-ml serologic pipette. (11) Glass stirring rod. (12) 37.5°C. water bath. (13) Stop watch. (14) Refrigerated centrifuge.

Procedure: In order to have a plasma-distilled water ratio of 1:19 when measuring whole-blood euglobulin lysis time, as in the plasma euglobulin lysis time, a correction is made for the plasma content of whole blood (estimated hematocrit 45 per cent). Thus 1.3 ml. of freshly drawn, unclotted whole blood (9 ml blood plus 1.0 ml. of 3.8 per cent sodium citrate) is *immediately* added to 13.5 ml. of distilled water in an iced Lusteroid test tube. While the tube is kept on ice, CO_2 gas is slowly bubbled through it for 3 minutes. In an alternate method, 1.3 ml. of the same blood is added to 13 5 ml of distilled water containing 0.22 ml. of 1 0 per cent acetic acid The tubes are left in the ice bath for 7 minutes and then spun in the refrigerated centrifuge at 4000-10,000 rpm for 10 minutes. The supernatant is decanted thoroughly (to get rid of contaminating inhibitors) and the precipitate reconstituted with 0.7 ml. S.P. buffer. Stirring with a glass rod renders the precipitate completely soluble. Seven-tenths ml of reconstituted euglobulin is transferred to a 12 × 75-mm. test tube and clotted with 0.05 ml. (2 N.I.H units) of thrombin. The tube is placed in the water bath and examined for lysis every 15 minutes during the first 2-3 hours, then less frequently Similar clots are made using 0.6 ml S P. buffer and 0.1 ml $2\ 8 \times 10^{-3}$ M EACA to reconstitute the clot (final EACA concentration is 4×10^{-4} M).

Interpretation The whole-blood euglobulin clot is more sensitive to both activator and plasmin than the plasma euglobulin clot, lysing in less than 20 minutes when an active system is present. If lysis is inhibited by 4×10^{-4} M EACA, the activity is assumed to be due to the presence of activator in the blood. If lysis occurs, uninhibited by EACA, it is due primarily to plasmin. In normal persons lysis of a whole-blood euglobulin clot requires at least 1½ hours *without* and 3½ hours *with* EACA.

Precautions and Sources of Error. (1) The euglobulin should be precipitated as soon as the blood is drawn, to permit measurement of activity before any loss occurs from enzyme lability (2) The rapidity of whole-blood euglobulin clot lysis is dependent upon both the activity present and

the fibrinogen content of the clot. Therefore, it is necessary to know the approximate fibrinogen content in order to interpret the results accurately. (3) Low fibrinogen samples are characterized by a small precipitate, inadequate co-precipitation of activator and plasmin, and poor clot formation.

8. Fibrinolytic Assay for Plasmin, Purified or in Biological Fluids (A. J. Johnson and A. O. Tse)

Object of Method: To determine the amount of plasmin in purified systems and biological fluids.

Principle underlying the Method: When plasmin is mixed in the cold with fibrinogen and thrombin and incubated at 37 5°C, the resulting clot lysis time is inversely proportional to the amount of plasmin that is present (under standard conditions of time, temperature, pH, ionic strength, type of buffer, etc [15,16]). The amount of plasmin which is required to lyse a standard clot in 10 minutes has been designated as 1 unit.

Reagents and Apparatus: (1) Sample containing plasmin (2) Bovine fibrinogen dissolved in S P. buffer, Reagent Table, page 461. (3) Working solution rabbit thrombin, Reagent Table, page 461. (4) S P. buffer (5) 25 per cent glycerol in S P. buffer. (6) Micrometric syringe buret (Model SB2, Micrometric Instrument Co., Cleveland, Ohio) (7) 0 2, 1.0, 2 0, 5.0, and 10 0-ml. serologic pipettes. (8) 12 × 75-mm test tubes (9) Ice bath (10) 37.5°C. water bath (11) Stop watch

Procedure: (a) Test tubes containing up to 0 7 ml S.P buffer are placed in an ice bath and (b) serial 0 05 log dilutions of plasmin with 25 per cent glycerol in S P. buffer are added with micrometric syringe buret. (c) Two-tenths ml fibrinogen, and (d) 0 1 ml thrombin are added to make final clot volume of 1 0 ml. The tubes are agitated at once and placed in a water bath, where they clot, and the stop watch is started.

Calculations: Clot lysis times are plotted against the reciprocals of the final dilutions of plasmin on log-log paper (fig. 4) (A French curve may be used, if necessary, to construct curve of best fit) Alternatively, a straight line graph may be obtained by plotting the reciprocal of the lysis time against the reciprocal of the final dilutions of the sample on a semi-log plot The dilution lysing the clot in 10 minutes (as indicated by the 10-minute intercept on the curve) is designated as 1 unit of plasmin The reciprocal of this dilution equals the number of units/ml in the sample being tested

Precautions and Sources of Error In fibrinolytic assays for plasmin, small amounts of human or bovine plasminogen in the fibrinogen or thrombin will not give erroneous results if test sample contains no activator.

Christensen Fibrinolytic Units/ml
Standard Plasmin Lot #8

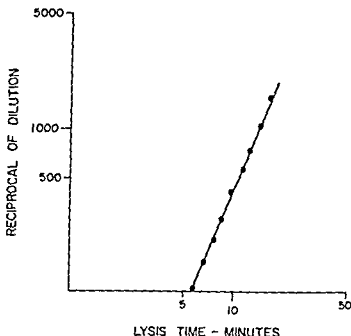


FIG 4—A typical fibrinolytic assay curve for plasmin, plasminogen, urokinase, or streptokinase (Ordinate is logarithmic plot of reciprocals of final dilutions, abscissa is logarithmic plot of lysis times)

Mean: 106; S.D.: ±8 5; coefficient of variation: ±8 per cent; standard error: ±1.8.

No of assays: 22.

9. Caseinolytic Assay for Plasmin Activity in Purified Plasmin Preparations or Biological Fluids (A. J. Johnson and A. O. Tse)

Object of Method: To determine the amount of plasmin in purified plasmin or biological fluids

Principle Underlying the Method: Purified alpha casein[17] is digested by plasmin, with a resulting increase in acid-soluble peptides (not precipitable with perchloric acid). Alpha casein was used instead of Hammersten's [illegible] because of its lower blank with perchloric acid precipitation, optical [illegible] to plasmin diges- [illegible] he initial reaction velocity of the digestion mixture is obtained by frequent serial sampling

during the first 30 minutes of incubation.[18] The released acid-soluble peptides are quantitated by direct spectrophotometry at 275 mμ[4] and read as the increase in O.D. of the released tyrosine/ml. sample/min. incubation.

Reagents and Apparatus: (1) Sample of purified plasmin or biological fluid. (2) 1.40 per cent solution of purified alpha casein in Tris-saline or saline-phosphate (S P.) buffer, pH 7.5 (solution may be frozen and stored). (3) Tris-saline or S P. buffer. (4) 1.5 M perchloric acid. (5) 15 × 100-mm. test tubes. (6) 0 2, 1.0, 2.0, 5.0 and 10.0-ml. serologic pipettes. (7) Stop watch. (8) 37.5°C. water bath. (9) #542 Whatman filter paper, 9-cm diameter. (10) Glass funnels, 50-mm. diameter with 65 mm stem. (11) Spectrophotometer with ultraviolet light source.

Procedure. (a) Thirteen ml. of Tris-saline or S. P. buffer dilution of purified plasmin or biological fluid is added to 13 ml. casein and incubated in a test tube in water bath (b) A separate blank is prepared at 0 time and samples are taken at intervals of 7, 10, 15, 20, 25 and 30 minutes; 4 ml. aliquots are removed and added to 1 ml. of perchloric acid in each tube The "0" time blank is prepared by adding separately 2 ml each of plasmin dilution and casein solution to 1 ml. perchloric acid. After allowing 1 hour for precipitates to form, incubated samples are passed through filters previously washed with acid, and filtrates and are read against blank in the spectrophotometer at 275 mμ.

Expression of Results—Calculations: One unit of plasmin activity is defined as the amount of enzyme which liberates 1 μg of tyrosine per minute per ml of sample under the conditions described. 37.5°C, 0 7 per cent purified alpha casein, Tris-saline or saline phosphate buffer (pH 7 5), as determined by the optical density (O.D) of the acid-soluble supernatant.

The change in O D. produced by an increase in tyrosine concentration of 1 μg /ml is determined from a standard tyrosine cure (typically, 1 μg. Ty/ml. = 0 0073 O.D. at 275 mμ.) When plasmin is incubated with casein and 7-10 serial samples are precipitated with perchloric acid during the first 30 minutes of incubation, the O D of acid-soluble tyrosine is plotted against the incubation time for each sample to determine the rate of tyrosine released per minute (typical calibration curve in fig 5). Thus

$$\mu g \text{ Ty/min. digestion} = \frac{\text{change in O D.}}{\text{min}} \times \frac{1}{0\,0073}$$

or

$$\frac{\text{O D /Ty 275}}{\text{min.}} \times 137\,0$$

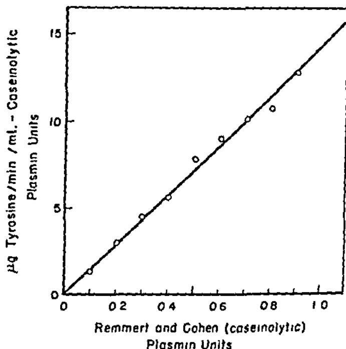


Fig. 5.—Conversion curve for plasmin Caseinolytic plasmin units (μg Tyrosine/min /ml) are plotted on ordinate against Remmert & Cohen units on abscissa

Therefore, units/ml plasmin in any given sample =

$$\frac{\text{O.D /Ty 275}}{\text{min.}} \times 137.0 \quad \times \text{dilutions, a, b, and c, where:}$$

a = dilution of sample prior to prep-

cipitation.

Under these conditions, one Remmert and Cohen[19] or Sgouris unit[20] of plasmin equals approximately 14 μg tyrosine released, or 14 plasmin units

Precautions and Sources of Error. (1) The range of linearity for the assay is 0.05-0.5 O.D. (2) The O.D. curve for the first 5 minutes may show a downward trend (due to alpha-1 casein) (3) Five to 10 serial samples of the digestion mixture should be assayed for precise determination of the linear part of the curve and initial reaction velocity. (4) #542 (or #42) Whatman filter paper should be rinsed with perchloric acid before

the sample is introduced, to remove extraneous substances which absorb at 275 mμ. Centrifugation will not remove all particulate matter. (5) All reagents should be at 37.5°C. when added, so that activation will begin immediately.

Caseinolytic Units/ml.

Standard Plasmin Lot #8

Mean: 140 (= 10 Remmert and Cohen units); S D.: ±10.5; coefficient of variation: ±7.5 per cent; standard error: ±1.75.

No of assays: 36.

10. Fibrinolytic Determination of Streptokinase (SK) or Urokinase (UK) Activity with Limited Activation of Human Plasminogen. (Method of L. R. Christensen, adapted by A. J. Johnson and A. O. Tse)

Object of Method: To determine the amount of SK or UK in purified systems or biological fluids.

Principle Underlying the Method· (a) When variable amounts of SK or UK are reacted for 10 minutes with a "fixed excess" of human plasminogen, the amount of plasmin formed (although not maximal) depends on the amount of SK or UK in the mixture. (b) When the mixture of SK or UK, with plasminogen, is added in the cold to fibrinogen and thrombin and incubated at 37 5°C , the resulting time of clot lysis is inversely proportional to the amount of plasmin that forms (under standard conditions of time, temperature, pH, ionic strength, type of buffer, etc)[21] (c) The amount of SK or UK (reflected as plasmin in the assay) which is required to lyse a standard clot in 10 minutes has been designated as 1 unit[1,21]

Reagents and Apparatus (1) Sample containing SK or UK (2) Dissolve bovine fibrinogen in S.P buffer, Reagent Table, page 461 (3) Partially purified human plasminogen in S P. buffer, Reagent Table, page 461 (4) Working solution rabbit thrombin, Reagent Table, page 461 (5) S.P. buffer. (6) Gelation S.P. buffer. (7) Micrometric syringe buret, (Model SB2, Micrometric Instrument Co , Cleveland, Ohio). (8) 0 2, 1 0, 2 0, 5 0 and 10 0-ml. serologic pipettes (9) 12 × 75-mm test tubes (10) Ice bath. (11) 37.5°C water bath (12) Stop watch

Procedure: (a) Test tubes containing up to 0 20 ml S P. buffer are placed in an ice bath and (b) serial 0 05 log amounts of SK or UK in gelation S P. buffer are added with the micrometric syringe buret (c) One-half ml purified human plasminogen, (d) 0 2 ml fibrinogen, and (e) 0 1 ml thrombin are added to make a 1.0-ml clot. The tubes are then shaken and placed in the water bath, and a stop watch is started

Calculations· Clot lysis times, plotted on log-log paper, against reciprocals of final dilutions of SK or UK form a straight line (fig. 4). The

dilution lysing the clot in 10 minutes (as indicated by the 10-minute intercept on the curve) is designated as 1 unit of either SK or UK. The reciprocal of this dilution equals the number of units/ml. of SK or UK in the sample being tested.

Comments: (1) The assay represents a limited modification of the Christensen assay[21] as used by Lederle Laboratories and the N.I.H. Division of Biologic Standards. (2) Limited activation of the plasminogen occurs during the period of clot formation, and during fibrinolysis; i.e., the assay is strongly dependent on the kinetics of plasminogen activation. (3) Small changes in plasminogen concentration effect a marked change in the assay result.

Christensen Fibrinolytic Units/vial

Standard SK Lot #2200-970A.

Mean: 100,000; S.D.: ±3,000; coefficient of variation: ±3 per cent; standard error: ±655

No. of assays: 21.

Standard UK Lot #1643-291

Mean: 18; S.D.: ±0.92; coefficient of variation: ±5 per cent; standard error: ±0.03.

No. of assays: 20.

Standard UK Lot # Trm-D-137

Mean: 33; S.D.: ±3; coefficient of variation: ±9 per cent; standard error: ±0.95.

No. of assays: 20.

10A. Fibrinolytic Determination of Streptokinase (SK) or Urokinase (UK) Activity with Maximal Activation of Human Plasminogen (A. J. Johnson and A. O. Tse)

Object of Method: To determine the amount of SK or UK in purified systems or biological fluids.

Principle Underlying the Method: Maximal amounts of plasmin are formed after prolonged pre-incubation of SK or UK with an optimal amount of human plasminogen.[22] The plasmin which is formed digests fibrin in a standard clot system (see Assay Method 8, page 467). For a similar method using casein as indicator substrate, see Assay 11, page 467.

Reagents and Apparatus: (1) Sample of SK or UK, purified or in biological fluids. (2) Tris-saline or saline phosphate (S.P.) buffer. (3) 50 per cent glycerol in Tris-saline or S.P. buffer. (4) Partially purified human plasminogen (5625 units/ml. or 18.75 mg. fraction III/ml. Tris-saline or S.P. buffer), standard reference lot No. 1897 or equivalent. (5) Reagents for plasmin fibrinolytic assay as described in Assay Method 8, page 467 (plasminogen-free fibrinogen is desirable with UK).

Procedure: (a) After making dilutions of SK or UK or biological fluid in Tris-saline or S.P. buffer, (b) 1.5 ml. sample dilution and 12 ml. human plasminogen are pre-incubated for 5 minutes at 37.5°C. (c) The plasmin is stabilized by the addition of 1.5 ml. 50 per cent glycerol in Tris-saline or S.P. buffer (final concentration 5 per cent) and the mixture is pre-incubated for another 15 minutes (a total of 20 minutes) (d) The plasmin is diluted with glycerol Tris-saline or glycerol S.P. buffer and assayed using fibrin as indicator substrate, as described in Assay Method 8, page 467 (lysis time range 4-40 minutes with reference plasminogen #1897). The optimal conditions for assay are 4500 fibrinolytic units of plasminogen (15 mg., #1897) per 5-15 fibrinolytic units of SK when the plasmin has been diluted approximately 50 times with glycerol Tris-saline or glycerol S.P buffer, although the range of SK may extend from 0.5-50 units with varied, appropriate dilutions of plasmin. The final concentrations of SK and plasminogen are important in performance of the assay. For a similar method, using casein as indicator substrate, see Assay 11, page 474

Calculations: A standard curve is made with 0.5-20 units of SK. When the clot lysis times are plotted on log-log paper against the reciprocals of the final dilutions of SK or UK, the curve is biphasic, one limb extending from approximately 2-10 units, the other from 10-50 units. Alternatively, a straight line is formed when the reciprocals of the lysis times are plotted against the reciprocals of the final dilutions of SK and UK on semi-log paper (fig. 6).

Comments: (1) In this assay, maximal activation of plasminogen occurs at a plasminogen level which represents true substrate excess (end product is plasmin only) (2) The assay is unaffected by small changes in plasminogen concentration (3) *Results may be compared directly with SK or UK Assay in Method 11, (fig 11) page 474 using casein as indicator substrate.* (4) Tris-saline buffer should be used consistently throughout the casein assay because the calibration curve with tris-saline is similar to but not directly applicable to the curve with S.P Tris-saline may also be used for the incubation mixture (activator + plasminogen) and S. P for the standard clot system because the clot is firmer and easier to visualize with S.P.

Christensen Fibrinolytic Units/vial

Standard SK Lot #2200-970A.

Mean: 600,000; S.D. ±30,000, coefficient of variation ±5 per cent, standard error: ±6000.

No. of assays 25

Standard UK Lot #1643-291

Mean: 300; S.D: ±16.5; coefficient of variation: ±5.5 per cent; standard error: ±3.3

dilution lysing the clot in 10 minutes (as indicated by the 10-minute intercept on the curve) is designated as 1 unit of either SK or UK. The reciprocal of this dilution equals the number of units/ml. of SK or UK in the sample being tested.

Comments: (1) The assay represents a limited modification of the Christensen assay[21] as used by Lederle Laboratories and the N.I.H. Division of Biologic Standards. (2) Limited activation of the plasminogen occurs during the period of clot formation, and during fibrinolysis: i.e., the assay is strongly dependent on the kinetics of plasminogen activation. (3) Small changes in plasminogen concentration effect a marked change in the assay result.

Christensen Fibrinolytic Units/Vial

Standard SK Lot #2200-970A.

Mean: 100,000; S.D.: ±3,000; coefficient of variation: ±3 per cent; standard error. ±655

No. of assays. 21.

Standard UK Lot #1643-291

Mean. 18; S.D.: ±0.92; coefficient of variation: ±5 per cent; standard error: ±0.03.

No. of assays: 20.

Standard UK Lot # Trm-D-137

Mean: 33, S.D.: ±3; coefficient of variation: ±9 per cent; standard error: ±0.95.

No. of assays: 20.

10A. *Fibrinolytic Determination of Streptokinase (SK) or Urokinase (UK) Activity with Maximal Activation of Human Plasminogen (A. J. Johnson and A. O. Tse)*

Object of Method. To determine the amount of SK or UK in purified systems or biological fluids.

Principle Underlying the Method Maximal amounts of plasmin are formed after prolonged pre-incubation of SK or UK with an optimal amount of human plasminogen.[22] The plasmin which is formed digests fibrin in a standard clot system (see Assay Method 8, page 467) For a similar method using casein as indicator substrate, see Assay 11, page 467.

Reagents and Apparatus (1) Sample of SK or UK, purified or in biological fluids. (2) Tris-saline or saline phosphate (S.P.) buffer. (3) 50 per cent glycerol in Tris-saline or S.P. buffer. (4) Partially purified human plasminogen (5625 units/ml or 18.75 mg. fraction III/ml. Tris-saline or S.P. buffer), standard reference lot No 1897 or equivalent. (5) Reagents for plasmin fibrinolytic assay as described in Assay Method 8, page 467 (plasminogen-free fibrinogen is desirable with UK).

Procedure: (a) After making dilutions of SK or UK or biological fluid in Tris-saline or S.P. buffer, (b) 1.5 ml. sample dilution and 12 ml. human plasminogen are pre-incubated for 5 minutes at 37.5°C. (c) The plasmin is stabilized by the addition of 1 5 ml. 50 per cent glycerol in Tris-saline or S P. buffer (final concentration 5 per cent) and the mixture is pre-incubated for another 15 minutes (a total of 20 minutes). (d) The plasmin is diluted with glycerol Tris-saline or glycerol S P. buffer and assayed using fibrin as indicator substrate, as described in Assay Method 8, page 467 (lysis time range 4-40 minutes with reference plasminogen #1897). The optimal conditions for assay are 4500 fibrinolytic units of plasminogen (15 mg, #1897) per 5-15 fibrinolytic units of SK when the plasmin has been diluted approximately 50 times with glycerol Tris-saline or glycerol S P. buffer, although the range of SK may extend from 0 5-50 units with varied, appropriate dilutions of plasmin. The final concentrations of SK and plasminogen are important in performance of the assay. For a similar method, using casein as indicator substrate, see Assay 11, page 474.

Calculations: A standard curve is made with 0 5-20 units of SK. When the clot lysis times are plotted on log-log paper against the reciprocals of the final dilutions of SK or UK, the curve is biphasic, one limb extending from approximately 2-10 units, the other from 10-50 units. Alternatively, a straight line is formed when the reciprocals of the lysis times are plotted against the reciprocals of the final dilutions of SK and UK on semi-log paper (fig 6).

Comments· (1) In this assay, maximal activation of plasminogen occurs at a plasminogen level which represents true substrate excess (end product is plasmin only). (2) The assay is unaffected by small changes in plasminogen concentration. (3) *Results may be compared directly with SK or UK Assay in Method 11, (fig 11), page 474 using casein as indicator substrate* (4) Tris-saline buffer should be used consistently throughout the casein assay because the calibration curve with tris-saline is similar to but not directly applicable to the curve with S P Tris-saline may also be used for the incubation mixture (activator + plasminogen) and S P for the standard clot system because the clot is firmer and easier to visualize with S P

Christensen Fibrinolytic Units/vial

Standard SK Lot #2200-970A

Mean· 600,000, S D.: ±30,000; coefficient of variation. ±5 per cent, standard error: ±6000

No of assays. 25

Standard UK Lot #1643-291

Mean: 300; S D : ±16.5; coefficient of variation. ±5 5 per cent; standard error. ±3 3.

dilution lysing the clot in 10 minutes (as indicated by the 10-minute intercept on the curve) is designated as 1 unit of either SK or UK. The reciprocal of this dilution equals the number of units/ml. of SK or UK in the sample being tested.

Comments: (1) The assay represents a limited modification of the Christensen assay[21] as used by Lederle Laboratories and the N.I.H. Division of Biologic Standards. (2) Limited activation of the plasminogen occurs during the period of clot formation, and during fibrinolysis: i e., the assay is strongly dependent on the kinetics of plasminogen activation. (3) Small changes in plasminogen concentration effect a marked change in the assay result.

Christensen Fibrinolytic Units/vial

Standard SK Lot #2200-970A.

Mean: 100,000; S.D : ±3,000; coefficient of variation: ±3 per cent; standard error: ±655.

No. of assays 21

Standard UK Lot #1643-291

Mean: 18; S D.: ±0.92; coefficient of variation: ±5 per cent; standard error: ±0 03

No. of assays: 20.

Standard UK Lot # Trm-D-137

Mean: 33; S.D.: ±3; coefficient of variation: ±9 per cent; standard error: ±0 95

No. of assays: 20

10A. Fibrinolytic Determination of Streptokinase (SK) or Urokinase (UK) Activity with Maximal Activation of Human Plasminogen (A. J. Johnson and A. O. Tse)

Object of Method· To determine the amount of SK or UK in purified systems or biological fluids

Principle Underlying the Method. Maximal amounts of plasmin are formed after prolonged pre-incubation of SK or UK with an optimal amount of human plasminogen.[22] The plasmin which is formed digests fibrin in a standard clot system (see Assay Method 8, page 467) For a similar method using casein as indicator substrate, see Assay 11, page 467

Reagents and Apparatus (1) Sample of SK or UK, purified or in biological fluids. (2) Tris-saline or saline phosphate (S P.) buffer (3) 50 per cent glycerol in Tris-saline or S P. buffer. (4) Partially purified human plasminogen (5625 units/ml or 18.75 mg. fraction III/ml. Tris-saline or [illegible] or equivalent. (5) Reagents [illegible] Assay Method 8, page 467 [illegible] UK).

Reagents and Apparatus: (1) Sample of SK or UK, purified or in biological fluid. (2) Tris-saline or saline phosphate (S P.) buffer. (3) 50 per cent glycerol in Tris-saline or S.P. buffer. (4) Partially purified human plasminogen (5625 units/ml. or 18.75 mg. fraction III Tris-saline or S P. buffer), standard reference lot No. 1897 or equivalent. (5) Reagents for plasmin casein assay as described in Method 9, page 468

Procedure: (a) Dilutions of a purified activator preparation, or biological fluid in Tris-saline or S P. buffer are made, and (b) 1.5 ml. of dilution and 12 ml. human plasminogen are pre-incubated for 5 minutes at 37.5°C. (c) The plasmin is stabilized by the addition of 1.5 ml. 50 per cent glycerol in Tris-saline or S.P. buffer (final concentration 5 per cent), and the mixture is pre-incubated for another 15 minutes (a total of 20 minutes). (d) An equal volume of 1.40 per cent alpha casein, as indicator substrate, is promptly added to form a plasmin digestion mixture which is assayed for plasmin as described in Method 9, page 468. (See fig 7 for a typical standard curve). The optimal conditions for assay are: 4500 fibrinolytic proactivator units of plasminogen (15 mg., #1897) per 2-20 fibrinolytic units of SK. The final concentrations of SK, plasminogen, and casein are important in the performance of the assay. Volumes of the reacting constituents are not critical although a total of 4 ml. is required for each perchloric precipitation to provide 2 ml for the spectrophotometer cuvette.

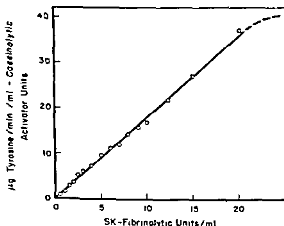


FIG 7—Conversion curve for SK assay with maximal activation of plasminogen Caseinolytic activator units (μg tyrosine/min/ml) are plotted on ordinate, and fibrinolytic SK units on abscissa (with limited activation of human plasminogen)

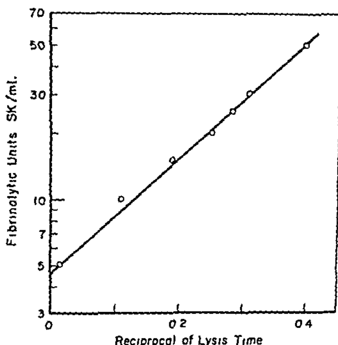


Fig 6.—Typical fibrinolytic SK or UK assay, with maximal activation of plasminogen Logarithm of reciprocals of final dilutions of sample are plotted on ordinate, and reciprocals of lysis times on abscissa

No. of assays. 25.

Standard UK Lot #Trm-D-137

Mean· 720; S D : ±43, coefficient of variation: ±6 per cent, standard error: ±9.

No of assays: 25.

11. *Caseinolytic Determination of Activator Activity of Streptokinase (SK) or Urokinase (UK) (A. J. Johnson and A. O. Tse)*

Object of Method. To determine the amount of free SK or UK present in human biological fluids or purified activator preparations

Principle Underlying the Method: Maximal amounts of plasmin are formed when SK or UK is pre-incubated, for an optimal period of time, with an excess of human plasminogen.[20] The plasmin which is formed [illegible] ulting increase [illegible] Assay Method [illegible] substrate, see [illegible] Assay Method 10A, page 472

Reagents and Apparatus: (1) Sample of SK or UK, purified or in biological fluid. (2) Tris-saline or saline phosphate (S.P.) buffer. (3) 50 per cent glycerol in Tris-saline or S P. buffer. (4) Partially purified human plasminogen (5625 units/ml. or 18 75 mg fraction III Tris-saline or S P. buffer), standard reference lot No 1897 or equivalent. (5) Reagents for plasmin casein assay as described in Method 9, page 468.

Procedure. (a) Dilutions of a purified activator preparation, or biological fluid in Tris-saline or S P. buffer are made, and (b) 1.5 ml. of dilution and 12 ml human plasminogen are pre-incubated for 5 minutes at 37.5°C (c) The plasmin is stabilized by the addition of 1.5 ml. 50 per cent glycerol in Tris-saline or S P. buffer (final concentration 5 per cent), and the mixture is pre-incubated for another 15 minutes (a total of 20 minutes). (d) An equal volume of 1.40 per cent alpha casein, as indicator substrate, is promptly added to form a plasmin digestion mixture which is assayed for plasmin as described in Method 9, page 468 (See fig 7 for a typical standard curve). The optimal conditions for assay are: 4500 fibrinolytic proactivator units of plasminogen (15 mg., #1897) per 2-20 fibrinolytic units of SK The final concentrations of SK, plasminogen, and casein are important in the performance of the assay. Volumes of the reacting constituents are not critical although a total of 4 ml is required for each perchloric precipitation to provide 2 ml. for the spectrophotometer cuvette

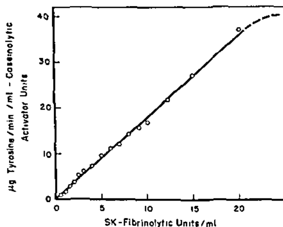


FIG 7—Conversion curve for SK assay with maximal activation of plasminogen Caseinolytic activator units (μg tyrosine/min/ml) are plotted on ordinate, and fibrinolytic SK units on abscissa (with limited activation of human plasminogen)

Calculations: The caseinolytic unit of activator is defined as that amount of enzyme which will release one caseinolytic unit of plasmin (1 µg. tyrosine per minute, per ml. of sample) under the conditions described. (See Assay Method 9 for plasmin unit and method).

Precautions and Sources of Error: (a) See Assay Method 9; (b) check plasminogen for contaminating plasmin (blank). (c) *Results may be compared directly with SK or UK assay in Method 10A, page 472, using fibrinogen as indicator substrate.* (d) Tris-saline *or* S.P. buffer should be used consistently throughout the assay because the calibration curve for one is similar to but not directly applicable to the other.

Caseinolytic Units/vial

Standard SK Lot #2200-970A.

Mean: 184,000 (= 13,143 Remmert and Cohen units); S.D.: ±15,000; coefficient of variation: ±8 per cent, standard error: ±2083.

No. of Assays: 52.

Standard UK Lot #1643-291

Mean: 2112 (= 151 Remmert and Cohen units); S.D : ±220.6; coefficient of variation: ±10 per cent, standard error: ±40.3.

No. of Assays: 30.

Standard UK Lot #Trm-D-137

Mean: 4050 (= 290 Remmert and Cohen units); S.D.: ±305.5; coefficient of variation ±7 5 per cent; standard error ±46 6

No. of Assays: 43.

12. *Fibrinolytic Determination of Plasminogen Proactivator in Human Purified Plasminogen or Biological Fluids* (A. J. Johnson and A. O. Tse)

Object of Method: To determine the number of plasminogen units in human purified plasminogen or biological fluids by a fibrinolytic activator assay utilizing bovine plasminogen and fibrinogen.[21,33]

Principle Underlying the Method: The addition of large amounts of SK to human purified plasminogen, or biological fluids containing plasminogen, results in the formation of SK-activator complex in amounts proportional to the amount of plasminogen. The complex is used to activate bovine plasminogen (which contains minimal proactivator) to plasmin Thus, when the complex is added to a fixed amount of bovine plasminogen, fibrin clot lysis occurs at a rate governed by the amount of SK activator present.[21 24]

Reagents and Apparatus (1) Sample of human purified plasminogen or biological fluid (2) SK (10,000 units/ml.) (3) Bovine fibrinogen, (Reagent Table, page 461), dissolved in Tris-saline buffer, and containing

70 units plasminogen and 0.45 mg. clottable protein per assay tube (reference lot No. 6211, Armour Pharmaceutical Co., Kankakee, Ill.). (4) Tris-saline buffer. (5) Working solution rabbit thrombin, Reagent Table, page 461. (6) 12 × 75-mm test tubes. (7) Micrometric syringe buret (Model SB2, Micrometric Instrument Co, Cleveland, Ohio). (8) 0 2, 1.0, 2 0, 5.0, and 10-ml. serologic pipettes (9) 37.5°C. water bath. (10) Ice bath (11) Stop watch.

Procedure: (a) Test tubes containing up to 0 60 ml. Tris-saline buffer are placed in an ice bath and (b) serial 0 05 log amounts of human purified plasminogen or biological fluid are added with micrometric syringe buret. (c) One-tenth ml. SK, (d) 0 2 ml. fibrinogen, and (e) 0.1 ml. thrombin are then added to make a final volume of 1 0 ml. The tubes are agitated at once, and placed in the water bath where they clot and a stop watch is started.

Calculations. Clot lysis times plotted against the reciprocal of the dilutions of plasma or plasminogen on log-log paper form a straight line (fig. 4). The final dilution lysing the clot in 10 minutes (as indicated by the 10-minute intercept on the curve) is said to contain 1 unit of plasminogen. The reciprocal of this dilution equals the number of units/ml. of plasminogen in the sample being tested. Reproducibility is ±10 per cent.

Precautions and Sources of Error: (a) The linearity of the curve extends from 7-17 minutes. (b) Contamination of plasminogen or bovine fibrinogen with plasmin, or of thrombin with plasmin or plasminogen will give erroneously high results; the plasmin contaminant may be detected by lack of stability during incubation of a standard clot with plasminogen at 37.5°C. The clot should be stable for at least 24 hours. (c) Plasma should be used for plasminogen assays because serum may give a lower value. (d) Samples containing little plasminogen with large amounts of inhibitor may lyse in reverse, i e., the more dilute sample may lyse first. Normally, inhibitors are diluted out

Christensen fibrinolytic Units/ml Plasma

Normal human plasma

Mean· 5000, S D ±215, coefficient of variation 4 4 per cent; standard error: ±48.1

No of Assays: 20.

13. Caseinolytic and Fibrinolytic Assays for Plasminogen (Proteolytic Precursor) in Human Purified Plasminogen Preparations or Biological Fluids (A. J. Johnson and A. O. Tse)

Object of Method. To determine the amount of plasminogen present in human purified plasminogen preparations or biological fluids.

Principle Underlying the Method: Complete conversion of plasminogen to plasmin occurs when SK is pre-incubated with human plasminogen in an optimal SK/plasminogen ratio.[18, 20, 22] The plasmin which is formed digests (a) purified alpha casein with a resulting increase in acid-soluble peptides on perchloric acid precipitation (see Assay Method 9, page 468), or (b) plasminogen-free fibrinogen in a standard clot system (see Assay Method 8, page 467).

Reagents and Apparatus: (1) Sample of purified human plasminogen or biological fluid. (2) Streptokinase (500 units/ml. Tris-saline buffer). (3) 50 per cent glycerol in Tris-saline buffer. (4) Tris-saline buffer. (5) Reagents for plasmin casein assay as described in Assay Method 9. (6) Reagents for plasmin fiibrinolytic assay as described in Assay Method 8.

Procedure: (a) Dilutions of purified human plasminogen or biological fluid in Tris-saline buffer are prepared (b) 12 ml. sample dilution and 1.5 ml. SK (final concentration 50 units/ml. after glycerol in Tris-saline is added as noted below) are pre-incubated for 5 minutes at 37.5°C. to form plasmin (c) The plasmin is stabilized by adding 1.5 ml. 50 per cent glycerol in Tris-saline buffer (final concentration 5 per cent) and the mixture is pre-incubated for another 15 minutes (total of 20 minutes). (d) An equal volume of 1.40 per cent alpha casein, as indicator substrate, is promptly added to form a plasmin digestion mixture which is assayed for plasmin as described in Assay Method 9, page 468. Alternatively, the plasmin formed in the 20-minute pre-incubation mixture may be diluted with glycerol Tris-saline buffer and assayed by using fibrin as indicator substrate, as described in Assay Method 8, page 467. The optimal conditions for assay are: 50 units of SK per 100-3000 fibrinolytic units of plasminogen, although the range of plasminogen extends from 50-5000 units The final concentration of SK, plasminogen and casein are important in the performance of the assay Volumes of the reacting constitutents are not critical although a total of 4 ml is required for each perchloric precipitation to provide 2 ml. for the spectrophotometer cuvette

Calculations See Assay Methods 8 and 9, and figure 8 for typical standard curve.

Precautions and Sources of Error (a) See Assay Methods 8 and 9 (b) Check plasminogen for contaminating plasmin (blank)

Caseinolytic Units/mg Fraction III

Standard human Fraction III, Lot #1897

Mean: 3.5 (= 0 25 Remmet and Cohen units), S D . ±0 2; coefficient of variation: ±5.7 per cent, standard error: ±0 047.

No. of Assays: 20.

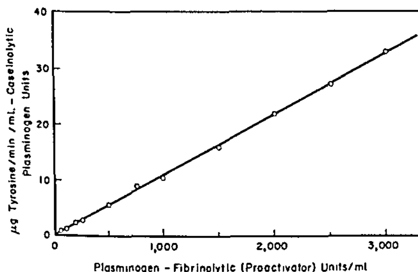


FIG 8—Conversion curve for plasminogen assay by the caseinolytic method Caseinolytic units of plasminogen (µg tyrosine/min /ml) are plotted on ordinate and fibrinolytic (proactivator) units on the abscissa

14. Fibrinolytic Estimation of Streptokinase (SK) Inhibitor and Antibody, and Urokinase (UK) Inhibitor in Purified Systems or Biological Fluids (A. J. Johnson, A. O. Tse and J. Newman)

Object of Method· To determine the amount of SK inhibitor and antibody, or UK inhibitor in purified systems or biological fluids [1]

Principle Underlying the Method The total SK inhibition assay employs dilutions of purified inhibitor, *serum*, or other biological fluid The UK inhibitor assay employs dilutions of (1) purified inhibitor, (2) serum, plasma, or other biological fluid. In each instance the dilutions are preincubated with 1 unit of SK or UK for 10 minutes to permit neutralization of part of the SK or UK by the inhibitor; the remaining free part is reacted with a standard amount of human plasminogen to lyse a standard bovine fibrin clot To increase accuracy of the assay and provide optimal association of enzyme and inhibitor, 50 per cent inhibition was chosen as the normal end point, i e , inhibition of 1 unit to ½ unit

Reagents and Apparatus (1) Sample of purified inhibitor, serum, plasma, or other biological fluid (2) Purified SK or UK (10 units/ml) (3) Dissolve bovine fibrinogen in S P buffer, Reagent Table, page 461 (5) Working solution rabbit thrombin, Reagent Table, page 461 (6)

Gelatin S.P. buffer, pH 7.5. (7) S.P. buffer. (8) Micrometric syringe buret. (9) 0.2, 1.0, 2.0, 5.0 and 10.0-ml. serologic pipettes. (10) 12 × 75-mm. test tubes. (11) Ice bath. (12) 37.5°C. water bath. (13) Stop watch.

Procedure: (a) Appropriate dilutions of a sample 0.1 ml volume, made with the micrometric syringe buret, are mixed with 0.1 ml. SK or UK (containing 1 unit) in test tubes which are placed in an ice bath for 10 minutes. Two controls (½ and 1 unit), in which S.P. buffer is substituted for the sample, are also placed in an ice bath (b) After the incubation period, 0.5 ml. of human plasminogen is added to each tube, then 0.2 ml. of bovine fibrinogen, and 0.1 ml. of thrombin to make a final clot volume of 1.0 ml. The tubes are agitated at once, placed in the water bath to clot and a stop watch is started.

Calculations: Clot lysis times are plotted against reciprocals of sample dilutions on a log-log plot (fig 9). A French curve may be used if necessary to construct curve of best fit. The reciprocal of the dilution having a lysis time equal to that of the ½ unit control (approximately 13 minutes, as

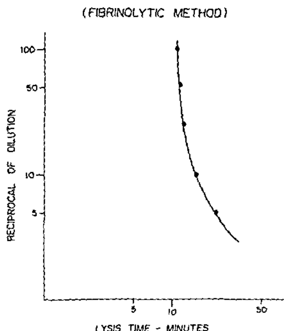


Fig 9.—Typical fibrinolytic assay of. plasmin, urokinase, streptokinase inhibitor, or streptokinase antibody Logarithms of reciprocals of final dilutions are plotted on the ordinate, and logarithms of the lysis times on the abscissa.

indicated by the intercept on the curve) is taken as the end point and represents the number of ½ units/ml. of sample. Divided by 2 it gives the number of units/ml. of sample. Calculations for UK inhibitor are the same as for SK. The range of linearity of the curve is 10-25 minutes; reproducibility is ±10 per cent.

Precautions and Sources of Error: (a) When spontaneous clotting of blood is delayed, the resulting plasma and serum mixture will give too low an assay result for total SK inhibitor. The assay must be repeated on the serum after coagulation is complete. (b) SK and UK inhibitors (not antibody) are temperature-labile. They should be kept on ice and assayed within 4-6 hours, or frozen immediately and assayed within 1 week.

15. *Fibrinolytic Determination of Plasmin Inhibitor in Purified Systems or Biological Fluids (A. J. Johnson, A. O. Tse and J. Newman)*

Object of the Method: To determine the amount of plasmin inhibitor in purified systems or biological fluids

Principle Underlying the Method: Dilutions of purified inhibitor or biological fluid are pre-incubated at room temperature with 1 5 units of glycerol-activated plasmin for a standard period of time Plasmin plus endogenous human plasmin inhibitor reaches equilibrium within 6-7 minutes,[1,15] while plasmin plus most of the purified plasmin inhibitors requires much less time. The neutralization mixtures are pre-incubated for 10 minutes at room temperature, because neutralization curves are asymptotic by this time. The mixture containing residual free plasmin is then added to standard bovine fibrinogen, clotted and the lysis time noted Fifty per cent inhibition was chosen as the end point, i e , inhibition of 1½ units of plasmin fibrinolytic activity (approximately 6½ minutes lysis time) to 1 unit of plasmin (10 minutes lysis time) One and one-half units of plasmin are used in this assay because the lysis time curve is relatively linear at this concentration

Reagents and Apparatus (1) Sample of purified inhibitor or biological fluid. (2) Purified glycerol-activated human plasmin (10 units/ml) (3) Bovine fibrinogen dissolved in S P buffer, Reagent Table, page 461. (4) Working solution of rabbit thrombin, Reagent Table, page 461 (5) 25 per cent glycerol in S.P. buffer, pH 7.5. (6) S P. buffer, pH 7 5 (7) Micrometric syringe buret. (8) 0.2, 1 0, 2.0, 5 0 and 10 0-ml serologic pipettes. (9) 12 × 75-mm. test tubes (10) 37.5°C water bath (11) Ice bath (12) Stop watch.

Procedure. (a) In the test tubes deliver. appropriate log dilutions of purified inhibitor or biological fluid, 0.15 ml plasmin (diluted with 25 per cent glycerol in S P. buffer), and S.P. buffer to a final volume of 0 7 ml. (b) The tubes are pre-incubated at room temperature for 10 minutes and

then placed in an ice bath. (Two 1-unit and one 1½-unit controls, in which S.P. buffer is substituted for the sample, are similarly incubated). (c) Two-tenths ml. bovine fibrinogen, and (d) 0.1 ml. thrombin are immediately added to each tube to form a standard clot. The final concentration of glycerol in the clot is approximately 5 per cent. The tubes are agitated at once, placed in the water bath to clot, and a stop watch is started.

Calculations: Lysis times are plotted against the reciprocals of the final dilutions of the sample on a log-log plot (fig. 9). A French curve may be used, if necessary, to construct curve of best fit. Alternatively, a straight line graph may be obtained by plotting reciprocals of lysis times against log of reciprocals of final dilutions of the sample on a semi-log plot The reciprocal of the dilution having a lysis time equal to that of the 1-unit control (as indicated by the intercept on the lysis time curve) is taken as the end point and represents the number of ½ units/ml in the sample. Divided by 2, it gives the number of units/ml sample.

Precautions and Sources of Error. (1) Different batches of glycerol plasmin vary with respect to their ability to neutralize endogenous plasmin inhibitor Therefore, the same lot number must be used for the patient's infusion and for the inhibitor assay. (2) On the other hand, equivalent fibrinolytic units of any lot of glycerol plasmin will be quantitatively neutralized by equal amounts of Kunitz pancreatic and soybean trypsin inhibitors, as well as other chemical inhibitors.

16. Chemical Determination of Epsilon Aminocaproic Acid (EACA) in Purified Systems or Biological Fluids (L. Skoza and A. J. Johnson)

Object of the Method· To determine by chemical means the amount of EACA in purified systems or biological fluids.[25]

Principle Underlying the Method (1) Basic and neutral amino acids and other reactive amines are pre-separated from acidic amino acids in deproteinized acidified biological fluids with ion exchange chromatography (Dowex 50), (2) the residual alpha amino acids are then removed from EACA by a copper carbonate–aluminum oxide column; and (3) EACA is determined spectrophotometrically by coupling with dinitrofluorobenzene (Sanger Reagent, or DNFB) (fig 10).[25]

Reagents and Apparatus (1) Plasma deproteinized by 50 per cent trichloroacetic acid (TCA) in the ratio of 1.0 ml. plasma to 0 2 ml 50 per cent TCA (2) 0 1 N HCl for acidification and dilution of protein-free biological fluids. (3) Cation exchange resin, analytical grade, Dowex AG 50W-X4 minus 400 mesh, hydrogen form (California Corp for Biochemical Research, Los Angeles) The resin is purified and cycled (Moore and Stein procedure[26]) until the eluate is colorless and no longer reacts with DNFB. It is then stored in excess water at 4°C. Prior to use it is filtered

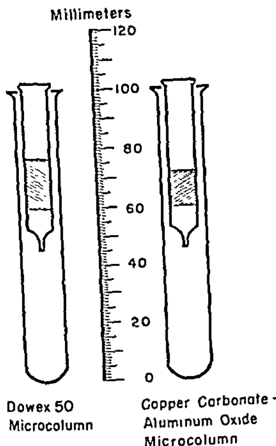


FIG 10—Comparative size of Dowex 50 microcolumn, used to separate basic and neutral amines and other reactive amines from the acidic amino acids, and the copper carbonate-aluminum oxide microcolumn used to separate residual alpha amino acids from EACA.

on sintered glass to remove free water and 0 615 ml water is added per Gm resin (4) Aluminum oxide (Al_2O_3), Woelm, non-alkaline (almost neutral), activity grade 1 for chromatographic analysis (M Woelm, Eschwege, Germany; U S distributors. Alupharm Chemicals, New Orleans) (5) 2, 4 dinitrofluorobenzene (Aldrich Chemical Co, Milwaukee), 0.2 ml DNFB in 10 ml. p-Dioxane, prepared fresh every day. (6) Cupric carbonate, Baker Analyzed Reagent Grade (J T. Baker Chemical Co., Phillipsburg, N.J.). (7) Yale glass syringes, 2 ml sterile, disposable (Becton Dickinson

and Co, Rutherford, N.J.). (8) International Clinical Centrifuge with horizontal swinging bucket rotor (Internation #221 or equivalent). (9) Spectrophotometer with moderate resolution at 430 mμ (Beckman DU or equivalent). (10) 15 × 100 mm. test tubes. (11) 65°C. water bath. (12) Stock solutions: 0.5 M NaH_2PO_4, pH 4.5; 0.1 M NaOH; 5 M HCl, 50 per cent trichloroacetic acid, 0.1 M NaH_2PO_4.

Preparation of Columns: Each of the Dowex 50 columns is composed of 1.4 ml. resin slurry in a disposable syringe. The dry copper-alumina (Cu-Al) column is a homogeneous mixture (1:3 by weight) of copper carbonate and aluminum oxide (see *Preparation of Reagents and Precautions and Sources of Error*). The mixture of Cu-Al is washed with water, dried at 85°C. overnight, and 0.9 Gm. is packed in each 2 ml. disposable syringe (fig 11). A sterile cotton wad is placed in the bottom of the syringe prior to use.

Procedure: The proteins and biological fluids are deproteinized and acidified for assay. Protein-free biological fluids are acidified with 0.1 M HCl. *Prior to use, 1.0 ml. distilled water is poured into the Dowex 50*

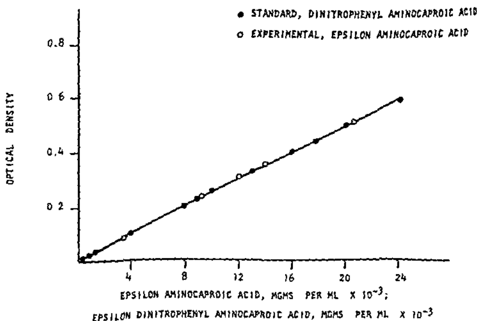


FIG. 11.—Quantitative, experimental recovery of epsilon aminocaproic acid followi the coupling reaction with dinitrofluorobenzene, plotted against optical density similar amounts of previously prepared, purified, epsilon dinitrophenyl aminocapro acid in 1M HCl

column and 2.4 ml. TCA filtrate is added while a thin layer of water remains on the resin surface. The column wall is then washed with 0.5 ml. distilled water to ensure complete adsorption of the sample. The column is eluted with approximately 5.5 ml. 0.5 M NaH_2PO_4 solution, pH 4.5, and centrifuged in a test tube (fig. 11) at 3000 r.p.m. for 5 minutes to eliminate excess buffer. The remaining amino acids are eluted by the addition of 2 0 ml , 0.1 N NaOH, and the columns and tubes are centrifuged as above for quantitative recovery. One and one-half ml of the eluate is adjusted to pH 8.5 ± 0 5 with exactly 1.5 ml. 0.1 M NaH_2PO_4 and applied to a dry Cu-Al column, which is slowly moistened with the first few drops. The column is centrifuged in a test tube at 1000 r.p m for 5 minutes to facilitate drainage.

One-tenth to 1.0 ml. of the pH 8.5 is reacted with 0.2 ml. DNFB reagent at 65°C. for 45 minutes. After it has been cooled on ice, 5 M HCl is added to a final volume of 2 ml. A blank is made by adding 1 5 ml 0.1 M NaH_2PO_4 to 1.5 ml. 0.1 M NaOH; it is reacted with DNFB under similar conditions. The optical density is read at 430 mμ (see fig. 12 for DNFB calibration curve).

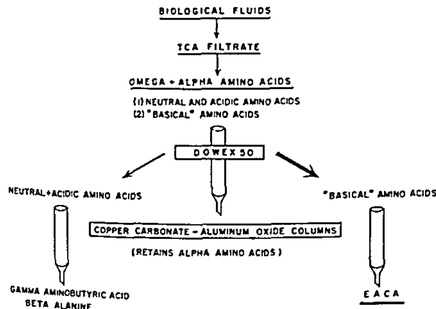


FIG. 12—Scheme for separation of EACA from biological fluids

Expression of Results—Calculations: $C_{EACA} = O.D._{430} \times dil. \times 17.6$ where:

C_{EACA} = concentration of EACA in ug /ml.,

$O.D._{430}$ = optical density reading at 430 mμ,

17.6 = factor derived from the graphical plot of $O.D._{430}$ *vs.* EACA-DNPh (epsilon-dinitrophenyl-aminocaproic acid),

dil. = over-all dilution of sample.

Normal Range of Values, Reproducibility: Five to 100 μg. ± 10 per cent. Normal blank = 0 7–2 μg. EACA equivalent.

Preparation of Reagents, Precautions and Sources of Error: (1) When the coupling yield of a known EACA solution is abnormally low, it is important to check the temperature, reaction time and pH of the coupling mixture (EACA-DNFB). (2) Each new or recycled batch of Dowex 50 must be calibrated for EACA binding capacity, using a known amount of EACA and varying amounts of 0.5 M NaH_2PO_4. The amount of phosphate eluate is constant for each batch but may range from 4–7 ml. in different batches. If too much phosphate eluate is used, some EACA is lost; if too little is used, the column's pre-separatory capacity is decreased The optimal amount is that which just precludes release of EACA from the column; i.e., when 6 ml. of phosphate starts the release of EACA, 5 ml. is used for pre-separation. (3) Each new batch of copper-alumina must be homogenized as an aqueous suspension, aged by drying at approximately 85°C overnight, and tested to ensure the quantitative passage of EACA through the column (without retention) The column is considered adequate if more than 1 2 mg. lysine and arginine is retained in 6 ml. diluent; if the effluent is not blue (due to copper-complexes of alpha amino acids); and if it permits the quantitative passage of more than 2 μg EACA in 2 5 ml. diluent. Unless these criteria are met, the aging procedure must be repeated. (4) When a plasma to which no EACA has been added shows a very high EACA equivalent value, either column may be at fault so both must be checked with known stock solutions of EACA (5) The method is applied to other biological systems with the following modifications: (a) urine is diluted at least 1/10 with distilled water before assay, and (b) 5 per cent TCA is used for deproteinization and simultaneous extraction of tissues, as well as red cells

E. COMPARATIVE VALUES OF STANDARD PREPARATIONS: CRITIQUE (A. J. Johnson and J. Newman)

It is evident from the data in tables 2–10 that many methods and many reagents are used to provide relatively accurate assays Since the results of these assays vary widely with respect to each other, they are extremely

difficult to interpret. Thus, it would seem to be necessary for assay results to be referred to a common standard at this time, although eventually, it would probably be desirable to have a greater uniformity of assay methods.

In general: (1) Any method must be reproducible (2) Uniform "standardized" reagents for each assay must be available in large amounts (preferably stockpiled). (3) Reagents must not be contaminated by plasmin (4) In fibrinolytic assays for human plasminogen (proactivator), bovine plasminogen (when present) must be very rigidly limited, or alternately may be added in great excess, as highly purified bovine plasminogen. When limited, as in the assay presented (Method #12), this can be readily accomplished by using a bovine fibrinogen preparation containing a precisely defined amount of plasminogen. (5) Potential *advantages* of the casein assay are: (a) reproducibility, (b) adaptability for determining and interpreting reaction rates, and (c) freedom from plasminogen contaminants However, assay results with the substrate casein cannot be equated with those obtained with the substrate fibrin because casein is not a physiologic substrate, and various plasmins differ markedly in the ratio of their ability to digest casein and fibrin A recently developed plasminogen-free fibrinogen with constant proteolytic reactivity may counter some of these objections.[56-52] (6) A "house," "national," or "international" standard must be used to evaluate assay results obtained by any method and to ensure reproducibility and constant potency of reagents. (7) In some earlier methods,[10,21,43] SK or UK was added to plasminogen in the presence of fibrin or casein as indicator substrate. Thus, activator formation and/or plasmin formation proceeded at a constant but somewhat inhibited rate concurrently with fibrinolysis or caseinolysis Comparable methods are distinguished (table 1) by the absence of a pre-incubation period for enzyme and substrate and have been found to have a great clinical utility in the thrombolytic treatment of a patient In general, they are sensitive, fast, easy to use, and are moderately reproducible. However, they require particularly well standardized reagents.

Other methods [19,20,27,37] have utilized pre-incubation of SK or UK with plasminogen, with maximal activation of the plasminogen to plasmin prior to the addition of indicator substrate (in great excess) Casein methods have been particularly well adapted in this regard, since a small amount of casein in the pre-incubation mixture tends to protect the newly formed plasmin These methods have been especially useful in the preparation of various components of the fibrinolytic system In general, they are less sensitive, a little more time-consuming, but yield very reproducible results, clearer kinetic data and can be used with a variety of purified reagents (with a suitable standard) An attempt has been made to combine the

Expression of Results—Calculations: $C_{EACA} = O.D._{430} \times dil. \times 17.6$ where:

C_{EACA} = concentration of EACA in ug./ml.,
$O.D._{430}$ = optical density reading at 430 mμ,
17.6 = factor derived from the graphical plot of $O.D._{430}$ *vs.* EACA-DNPh (epsilon-dinitrophenyl-aminocaproic acid),
dil. = over-all dilution of sample.

Normal Range of Values, Reproducibility: Five to 100 μg. ± 10 per cent. Normal blank = 0.7–2 μg. EACA equivalent.

Preparation of Reagents, Precautions and Sources of Error: (1) When the coupling yield of a known EACA solution is abnormally low, it is important to check the temperature, reaction time and pH of the coupling mixture (EACA-DNFB). (2) Each new or recycled batch of Dowex 50 must be calibrated for EACA binding capacity, using a known amount of EACA and varying amounts of 0.5 M NaH_2PO_4. The amount of phosphate eluate is constant for each batch but may range from 4–7 ml. in different batches. If too much phosphate eluate is used, some EACA is lost; if too little is used, the column's pre-separatory capacity is decreased. The optimal amount is that which just precludes release of EACA from the column; i.e., when 6 ml. of phosphate starts the release of EACA, 5 ml. is used for pre-separation (3) Each new batch of copper-alumina must be homogenized as an aqueous suspension, aged by drying at approximately 85°C. overnight, and tested to ensure the quantitative passage of EACA through the column (without retention). The column is considered adequate if more than 1.2 mg lysine and arginine is retained in 6 ml diluent; if the effluent is not blue (due to copper-complexes of alpha amino acids); and if it permits the quantitative passage of more than 2 μg EACA in 2.5 ml. diluent Unless these criteria are met, the aging procedure must be repeated (4) When a plasma to which no EACA has been added shows a very high EACA equivalent value, either column may be at fault so both must be checked with known stock solutions of EACA (5) The method is applied to other biological systems with the following modifications (a) urine is diluted at least 1/10 with distilled water before assay, and (b) 5 per cent TCA is used for deproteinization and simultaneous extraction of tissues, as well as red cells

E. COMPARATIVE VALUES OF STANDARD PREPARATIONS: CRITIQUE (A. J. Johnson and J. Newman)

It is evident from the data in tables 2–10 that many methods and many reagents are used to provide relatively accurate assays Since the results of these assays vary widely with respect to each other, they are extremely

Table 3. Variation in Results of Caseinolytic Assays on a Single Standard Glycerol-Activated Plasmin Preparation #3 (Tests Performed by a Variety of Technics, in Different Laboratories)

Investigator Number	Results Reported	Definition of Casein Unit	Indicator Substrate Used (final concentration)	House Standard	Reference For Method
2	29 units/ml	Δ1 O.D./min, 280 mμ, 30 min incubation, 37°C	2% casein (distilled water, alcohol, ether)	American Red Cross plasmin	1, 2, 3
5	6.94 units/ml	O.D. 650 tyrosine: 180 μg, 60 min	2% casein	none	4, 3
7	30.5 units/ml	180 μg tyrosine 60 min	2% casein	none	5, 1
8	9.5 units/ml	450 μg tyrosine 60 min	4% casein	none	6
11	15.2 units/ml	450 μg tyrosine 60 min	2% casein	none	3
12	10.3 units/ml	O.D. 280 tyrosine: 450 μg, 60 min	3% casein	none	7, 3
13	11.2 units/ml	O.D. 280 tyrosine: 450 μg, 60 min	2% casein (Müllertz purification)	Ortho plasmin	6
14	140 units/ml coefficient of variation ±7.5% (10 R&C units/ml)	O.D. 275 tyrosine: 1 μg/min	0.7% alpha casein	N.I.H.	8, 2, 9
15	11.1 units/ml	O.D. 280 tyrosine: 450 μg, 60 min	4% casein	N.I.H.	10
17	10.7 units/ml	O.D. 280 tyrosine: 450 μg, 60 min	4% casein	none	6
18	45.5 units/ml	Δ1 O.D./min, 280 mμ 60 min, 37.5°C	1.5% casein	UK-plasmin	11, 12
19	22.1 units/ml	O.D. 275 tyrosine: 1 μg/min	1.5% casein (Müllertz purification)	none	13, 12
20	10.1 units/ml	O.D. 280 tyrosine: 450 μg, 60 min	4% casein	N.I.H.	6

TABLE 2 *Variation in Results of Fibrinolytic Assays on a Single Standard Glycerol-Activated Plasmin Preparation #8 (Tests Performed by a Variety of Technics, in Different Laboratories)*

Investigator Number	Results Reported	Definition of Fibrinolytic Unit	Indicator Substrate Used (final concentration)	House Standard	Reference For Method
3	1.6 units/ml	lysis of clot, 2 min., 28°C.	0.1% freeze-thaw bovine fibrinogen	none	14
4	0.29 units/ml	amount of preformed plasma clot (expressed in µl.) lysed in 24 hr. at 37°C by 1 ml. of same plasma in which p-iodobenzoic acid Na has been dissolved to make a concentration of 0.074 M	human plasma clot	0.074 M p-iodobenzoic acid Na	15
9	283 units/ml	lysis of clot, 10 min., 37°C.	0.45% bovine fibrinogen	varidase	16
14	106 units/ml coefficient of variation ±8%	lysis of clot, 10 min., 37°C	0.45% bovine fibrinogen	varidase	16, 17
17	12 units/ml	lysis of clot, 2 min., 37°C.	0.3% human fibrinogen	Parke Davis plasmin	18
18	13.8 units/ml	lysis of clot, 2 min., 45°C.	0.3% human fibrinogen	UK plasmin	18
21	25.1 mg/min./ml	cold soluble fibrin released by one Remmert and Cohen unit/ml. = 2.51 mg/min.	0.08-0.5% human fibrinogen (plasminogen- and "inhibitor"-free)	N.I.H.	19

TABLE 5 *Variation in Results of Caseinolytic Assays on a Single Standard Streptokinase Preparation # 2200-970 4 (Tests Performed by a Variety of Technics, in Different Laboratories)*

Investigator Number	Results Reported	Definition of Unit	Indicator Substrate (final concentration)	Plasminogen (final concentration)	House Standard	Reference For Method
2	140,000 units per vial	Δ 1 O D /min , 280 mμ, 30 min , 37°C.	2% casein	guinea pig euglobulin	American Red Cross plasmin	1, 2, 3
7	100,000 units per vial	180 μg tyrosine/60 min , 37°C	2% casein	human plasminogen (10 casein units)	none	5, 1
11	198,000 units per vial	450 μg tyrosine/ 60 min.	2% casein	human plasminogen (4 3 casein units)	none	3
17	34,500 units per vial	450 μg tyrosine/ 60 min	4% casein	human plasminogen (11.5 casein units)	none	6
14	184,000 units per vial (13,143 R&C units per vial) coefficient of variation ±8%	O D 275 tyrosine 1 μg /min	0 7% alpha casein	human plasminogen (4500 Christensen units)	N I H	27, 9

TABLE 4 *Variation in Results of Fibrinolytic Assays on a Single Standard Streptokinase Preparation # 2200-970A (Tests Performed by a Variety of Technics, in Different Laboratories)*

Investigator Number	Results Reported	Definition of Unit	Indicator Substrate (final concentration)	Plasminogen (final concentration)	House Standard	Reference For Method
3	60,600 units per vial	lysis of clot, 2 min, 37°C.	0.1% freeze-thaw bovine fibrinogen	human plasminogen (approx. 9 casein units)	none	20, 21
4	1300 units per vial	amount of preformed clot (expressed in μl) lysed in 24 hours at 37°C by 1 ml of the same plasma containing p-iodobenzoic acid Na at a concentration of 0 074 M	fibrinogen in human plasma	human plasma	human plasma and p-iodobenzoic acid Na	15
8	100,000 units per vial	lysis of clot, 10 min, 37°C	approx 0 46% bovine fibrinogen (clottable protein)	approx 150 Christensen units	NIH reference No 2	16
9	100,000 units per vial	lysis of clot, 10 min, 37°C	0 46% bovine fibrinogen (clottable protein)	150 Christensen units human plasminogen	Varidase	16, 22
13	103,450 units per vial	lysis of clot, 10 min, 37°C	0 46% bovine fibrinogen (clottable protein)	150 Christensen units human plasminogen		16, 22
14	100,000 units per vial coefficient of variation ±3%	lysis of clot, 10 min, 37°C	0 46% bovine fibrinogen (clottable protein)	150 Christensen units human plasminogen	Varidase Ploug	16, 22
14	600,000 units per vial coefficient of variation ±5%	lysis of clot, 10 min, 37°C	0 46% bovine fibrinogen (clottable protein)	4500 Christensen units human plasminogen (pre-incubated)	Varidase Ploug	16, 23
15	100,000 units per vial	Modified Ploug unit	bovine fibrinogen			24
16	96,000 units per vial	lysis of clot, 2 min., 37°C.	0 30% human fibrinogen	0 045% human Cohn fraction III	Ploug	25, 26
17	556,700 units per vial	lysis of clot, 2 min, 45°C	0 3% human fibrinogen	4 R P.M I units of human plasminogen	Ploug	18
18	156,000 units per vial			1% human plasminogen	R P M I. standard	18

9	45 5 units per vial	lysis of clot, 10 min, 37.5°C.	0 46% bovine fibrinogen (clottable protein)	150 Christensen units human plasminogen	Varidase	16, 22
14	33 units per vial coefficient of variation ±9%	lysis of clot, 10 min, 37 5°C = 76 Ploug units	0 46% bovine fibrinogen (clottable protein)	150 Christensen units human plasminogen	Varidase Ploug	22, 16
14	720 units per vial coefficient of variation ±6%	Lysis of clot, 10 min, 37.5°C.	0 46% bovine fibrinogen	4500 Christensen units (pre-incubation)	Varidase Ploug	23, 16
15	58,500 units per vial					24
18	2340 units per vial	lysis of clot, 2 min, 45°C.	0.3% human fibrinogen	Human plasminogen	R P M I standard	18
22	22,700 units per vial coefficient of variation ±4 8%	approximately 8 6 units to 1 Ploug unit	0 2% bovine fibrinogen (clottable protein)	bovine plasminogen (varies with fibrinogen)	3 house standards	31, 32, 29

TABLE 6 *Variation in Results of Fibrinolytic Assays on a Single Standard Urokinase Preparation Trm-D-137 (Tests Performed by a Variety of Technics, in Different Laboratories)*

Investigator Number	Results Reported	*Definition of Unit*	Indicator Substrate *(final concentration)*	Plasminogen *(final concentration)*	House Standard	Reference For Method
1	1230 units per vial	Ploug unit	0 4% bovine fibrinogen	bovine (varies with fibrinogen)	Ploug	26
6	1690 units per vial	Ploug unit	0 1% bovine fibrinogen	0 25% bovine (Cohn fractionation)	Ploug	28
16	9600 units per vial	modified Ploug unit	0.25% bovine fibrinogen	0 045% human Cohn fraction III	Ploug	25, 26
17	2854 units per vial	lysis of clot, 2 min, 37°C	0 3% human fibrinogen	4 R P.M.I units of human plasminogen	Ploug	18
19	2340 units per vial	Ploug	0 2% bovine fibrinogen	bovine (varies with fibrinogen)	Ploug	29
10	3100 units per vial	½ unit = 50% lysis in 17 hr, 37 5°C	0 38% bovine fibrinogen (clottable protein)	bovine (varies with fibrinogen)	Abbott UK	30, 26
3	149 2 units per vial	lysis of clot, 2 min, 37°C	0 1% freeze-thaw bovine fibrinogen	bovine (varies with fibrinogen)		20, 21
4	1 7 units per vial	amount of preformed plasma clot (expressed in μl) lysed in 24 hr. at 37°C by 1 ml of the same plasma containing dissolved p-iodobenzoic acid Na at a concentration of 0 074 M	fibrinogen in human plasma	human plasma	plasma human + p-iodobenzoic acid	15

TABLE 8 *Variation in Results of Fibrinolytic Assays on a Single Standard Urokinase Preparation #1643-291 (Tests Performed by a Variety of Technics, in Different Laboratories)*

Investigator Number	Results Reported	Definition of Unit	Indicator Substrate (final concentration)	Plasminogen (final concentration)	House Standard	Reference For Method
1	710 units per vial	Ploug unit	0 4% bovine fibrinogen	bovine (varies with fibrinogen)	Ploug	26
6	770 units per vial	Ploug unit	0 1% bovine fibrinogen	0 25% bovine (Cohn fractionation)	Ploug	28
16	4000 units per vial	modified Ploug unit	bovine fibrinogen	0 045% human Cohn fraction III	Ploug	25, 26
17	959 units per vial	lysis of clot, 2 min, 37°C	0 3% human fibrinogen	4 R P M I. units of human plasminogen	Ploug	18
19	1000 units per vial	Ploug unit	0 2% bovine fibrinogen	bovine (varies with fibrinogen)	none	29
3	132 units per vial	lysis of clot, 2 min, 37°C	0 1% freeze-thaw bovine fibrinogen	bovine (varies with fibrinogen)		20, 21
4	0 32 units per vial	amount of preformed plasma clot (expressed in µl) lysed in 24 hours at 37°C by 1 ml. of same plasma containing dissolved p-iodobenzoic acid Na at a concentration of 0 074 M	fibrinogen in human plasma	human plasma	human plasma and p-iodobenzoic acid Na	15

(*Table 8 is continued on p 496*)

TABLE 7 *Variation in Results of Caseinolytic Assays on a Single Standard Urokinase Preparation Trm-D-137 (Tests Performed by a Variety of Technics, in Different Laboratories)*

Investigator Number	Results Reported	Definition of Unit	Indicator Substrate (final concentration)	Plasminogen (final concentration)	House Standard	Reference For Method
7	369 5 units per vial	180 μg tyrosine/60 min, 37°C	2% casein	human plasminogen (10 units)		5, 1
11	2285 units per vial	450 μg tyrosine/60 min	2% casein	human plasminogen (4 3 units)		3
13	1165 units per vial	O D 280 tyrosine 450 μg /60 min	2% casein (Mullertz purification)	human plasminogen (20 casein units)	house standard	6
17	484 units per vial	450 μg tyrosine/60 min.	4% casein	human plasminogen (11.5 casein units)	none	6
14	4050 units per vial	O D 275 tyrosine 1 μg /min	0 7% alpha casein	human plasminogen (4500 Christensen units)	Ploug	27, 6, 2
	coefficient of variation ±7 5% (290 R&C units per vial)					

TABLE 9 *Variation in Results of Caseinolytic Assays on a Single Standard Urokinase Preparation # 1643-291 (Tests Performed by a Variety of Technics, in Different Laboratories)*

Investigator Number	Results Reported	Definition of Unit	Indicator Substrate (final concentration)	Plasminogen (final concentration)	House Standard	Reference For Method
2	146 units per vial	1 O D /min , 280 mμ, 30 min , 37°C.	2% casein	guinea pig euglobulin	American Red Cross plasmin	1, 2, 3
7	170 units per vial	180 μg. tyrosine/ 60 min , 37°C.	2% casein	human plasminogen (10 casein units)	none	5, 1
11	1250 units per vial	450 μg tyrosine/ 60 min	2% casein	human plasminogen (4 3 casein units)	none	3
13	544 units per vial	O D 280 tyrosine 450 μg /60 min.	2% casein (Mullertz purification)	human plasminogen (20 casein units)	house standard	6
17	241 units per vial	450 μg. tyrosine/ 60 min	4% casein	human plasminogen (11.5 casein units)	none	6
14	2112 units per vial 151 R&C units per vial coefficient of variation ±10%	O D 275 tyrosine. 1 μg /min	0.7% alpha casein	human plasminogen (4500 Christensen units)	Ploug	27, 9, 6

TABLE 8 *Variation in Results of Fibrinolytic Assays on a Single Standard Urokinase Preparation #1643-291 (Tests Performed by a Variety of Technics, in Different Laboratories)*

Investigator Number	Results Reported	Definition of Units	Indicator Substrate (final concentration)	Plasminogen (final concentration)	House Standard	Reference For Method
9	20 units per vial	lysis of clot, 10 min, 37 5°C.	0 46% bovine fibrinogen (clottable protein)	150 Christensen units human plasminogen	Varidase	16, 22
10	1900 units per vial	½ unit = 50% lysis in 17 hours, 37 5°C	0 38% bovine fibrinogen (clottable protein)	bovine (varies with fibrinogen	Abbott UK	30, 26
14	18 units per vial coefficient of variation ±5%	lysis of clot, 10 min, 37 5°C = 76 Ploug units	0 46% bovine fibrinogen (clottable protein)	150 Christensen units human plasminogen	Varidase	16, 22
14	300 units per vial coefficient of variation ±5 5%	lysis of clot, 10 min, 37 5°C.	0 46% bovine fibrinogen (clottable protein)	4500 Christensen units (pre-incubation)	Varidase Ploug	23, 16
15	18,600 units per vial					24
18	100 units per vial	lysis of clot, 2 min, 45°C	0 3% human fibrinogen	0 5% human plasminogen (pre-incubation)	R.P M.I. standard	18
22	8810 units per vial coefficient of variation ±5 3%	approximately 8 6 units to 1 Ploug unit	0 2% bovine fibrinogen (clottable protein)	bovine plasminogen (varies with fibrinogen)	3 house standards	31, 32, 29

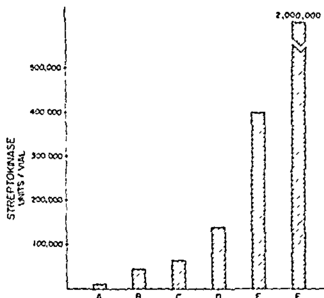


FIG. 11—Results of fibrinolytic assays of one vial of standard, high-purity SK (designated as 100,000 units) with reagents varying in origin and purity but having similar activity. For example, all thrombin preparations produced identical coagulation times with test fibrinogen. (A) Human fraction III,* bovine fraction I,† rabbit thrombin,‡ (B) Human fraction III,§ bovine fraction I,† rabbit thrombin,‡ (C) Human fraction III,§ bovine fibrinogen,|| rabbit thrombin,‡ (D) Human fraction III,§ bovine fibrinogen,|| human thrombin,** (E) Human fraction III,§ human fibrinogen,†† human thrombin,** (F) Human plasminogen,†† human fibrinogen,‡‡ human thrombin **

* Lot 714, Lederle Laboratories, division of American Cyanamid Co, Pearl River, N Y

† Lot U4977, Armour Pharmaceutical Co., Kankakee, Ill

‡ Lot 308, plasminogen-free (former N I.H Standard for SK Assay), Lederle Laboratories

§ Lot 1893A, collected and pooled by the American National Red Cross, and processed by E. R. Squibb & Son, New York, N Y

|| High purity, plasminogen-free, prepared by Method 5 p 000

** High purity, plasminogen-free, prepared by Dr Kent Miller, N Y State Health Department Laboratories, Albany, N Y

†† High purity, plasminogen-free, prepared by Research Laboratories of the American National Red Cross

‡‡ High purity, prepared by Dr. Per Wallen, Karolinska Inst., Stockholm, Sweden

TABLE 10 *Conversion Table for Standard UK Preparations*

[illegible]	≈ 950 CTA units*
[illegible]	= 1800 CTA units*
[illegible]	= 2900 CTA units*
1 CTA unit	≈ 2 0 Abbott units
	= 12 4 Sterling-Winthrop units
	= 0 75 Leo units (approximate)
Ratio of total activity in vials (SW/Abbott)	≈ 1 9†
Ratio of relative activity in units of manufacturers (SW/Abbott)	≈ 6 2†

* These standardization data are published by the courtesy of the Committee on Thrombolytic Agents of the United States Public Health Service, National Heart Institute The values stated are approximate and do not represent official National Institutes of Health standards

† Rates confirmed by fibrinolytic, caseinolytic and esterolytic methods

virtues of both methods in sections 10A, 11, and 13 by pre-incubation of SK or UK with plasminogen, stabilization of the resulting plasmin with glycerol, and addition of this plasmin to both highly purified alpha casein and a plasminogen-poor fibrin clot system as indicator substrates.

For many reasons, it is most desirable that fibrinolytic assays of enzymes used to produce thrombolysis in man be performed with highly purified human reagents However, their purification poses formidable problems, e.g , increasing purity is accompanied by an apparent increase in fibrinolytic activity in the standard assay. This increase occurs with plasminogen-free reagents (fig. 13) and cannot be due to plasminogen contamination of the reagents under these circumstances. Furthermore, slight variations in highly purified human reagents such as thrombin[53] produce highly variable assay results (fig. 14). Since reproducibility of an assay is a basic requisite for standardization, and since highly purified, biophysically homogeneous reagents may differ in their sensitivity to plasmin, maximal purification of the human reagents must include reproducible sensitivity and accuracy in the assay system (fig 15).

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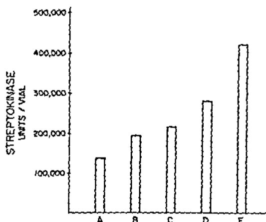


FIG 14.—Results of fibrinolytic assays on one vial of standard, high-purity SK (*designated as 100,000 units*) *with five different preparations of plasminogen-free* thrombin of similar activity. Human fraction III and human, high-purity, plasminogen-free fibrinogen were used *throughout* (*A*) Rabbit thrombin (former N.I.H. standard for SK assay), Lederle Laboratories, division of American Cyanamid Co., Pearl River, N.Y. (*B-E*) *High-purity human thrombins*, prepared by Dr. Kent Miller, N.Y State Department of Health Laboratories, Albany, N.Y.

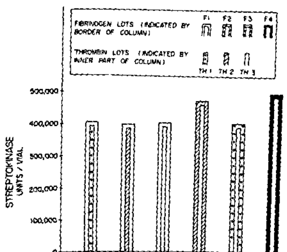


FIG 15.—Results *of fibrinolytic* assays of one vial of standard, *high-purity* SK (designated as 100,00 units) with one lot of *human fraction III* and *different* preparations of human, highly purified, plasminogen-free fibrinogen and thrombin of similar activity Because *reagents* are *highly purified*, results *show relatively* small variation.

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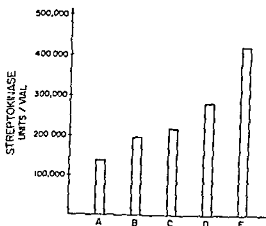


Fig 14.—Results of fibrinolytic assays on one vial of standard, high-purity S (designated as 100,000 units) with five different preparations of plasminogen-fre thrombin of similar activity Human fraction III and human, high-purity, plasmi ogen-free fibrinogen were used throughout. (A) Rabbit thrombin (former NIH standard for SK assay), Lederle Laboratories, division of American Cyanamid Co Pearl River, N Y. (B-E) High-purity human thrombins, prepared by Dr. Ken Miller, N.Y State Department of Health Laboratories, Albany, N.Y.

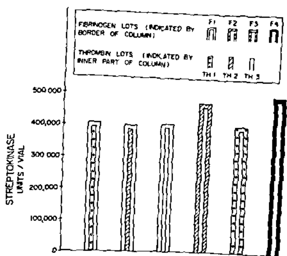


Fig 15.—Results of fibrinolytic assays of one vial of standard, high-purity SK (designated as 100,00 units) with one lot of human fraction III and *different* preparations of human, highly purified, plasminogen-free fibrinogen and thrombin of similar activity. Because reagents are highly purified, results show relatively small variation

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CHAPTER IV

STUDY OF INTRAVASCULAR COAGULATION

1. *Estimation of Intravascular Utilization of Coagulation Factors**

R. D. LANGDELL, W. P. WEBSTER and K. M. BRINKHOUS

Objective of Procedure: During the past several years, specific quantitative assay procedures have been developed for most of the plasma coagulation factors. This has made it possible to study the in vivo utilization of these factors by determining their rate of loss from the circulation after injection[1-3] Such information is of value in determining the physiologic activity of a plasma fraction and is essential in planning a therapeutic regimen with transfusions of whole blood, plasma, or plasma fractions for alleviation of procoagulant deficiencies. The procedure described furnishes an experimental design by which the plasma clearance of any of several procoagulants may be estimated.

Principle of the Test The intravenous administration of a plasma coagulation factor results in its increase in the blood of the recipient The maximal increase in activity is dependent upon the amount of material administered and occurs promptly after administration, provided that synthesis of the procoagulant is not stimulated in the recipient After injection, the activity in the plasma of the recipient decreases progressively with time The loss of activity from the plasma is the plasma clearance, the rate of which may be calculated.

Reagents and Equipment They are determined by the procoagulant under study (a) For the assay procedure· The reagents and equipment will be determined by the specific assay procedure used (see section on appropriate specific assay procedure) Sufficient volumes of all reagents should be on hand at the start of the study so that all determinations can be

* Investigations upon which this method is based were supported in part by research grants H-1648 and H-06350 and fellowship grant, GM-K3-15,091

performed with the same batch of reagents. If the subject's plasma is to be used as a substrate in the assay procedure, sufficient plasma should be collected and stored in proper aliquots at −20°C. prior to the beginning of the study. (b) Material for injection: This is usually citrated whole blood or plasma from a normal subject, or a concentrated plasma fraction rich in the procoagulant being studied.

Steps in Performance of Procedure: (*a*) *Selection of recipient:* The basic experimental design will determine the type of recipient to be used. In general, the recipient will be in one of the following categories: (1) Hereditary deficiency states. Patients with severe deficiencies of a single coagulation factor are often subjects for this type of study. Animals with a hereditary deficiency, such as hemophilic dogs, are ideal subjects Preliminary diagnostic studies must be done to characterize the nature and extent of the deficiency. If the deficiency is mild, extensive preliminary studies are essential to evaluate circumstances that might alter the circulating level of the factor before injection studies are undertaken (2) Acquired deficiencies. Occasionally the rare patient with an acquired isolated deficiency of a procoagulant is used Patients receiving one of the coumarin or indandione drugs who have multiple procoagulant deficiencies may be used Experimental animals with induced deficiencies are also employed In the acquired deficiencies the decrease in plasma procoagulant activity is reversible and the experiment must be carefully designed so that synthesis is kept constant during the post-infusion study. (3) Normal subjects

(*b*) *Study of recipient immediately prior to infusion* (1) Determine the plasma level of the factor under study prior to injection of the active material (2) Determine the blood volume of the subject Any standard method of measuring blood volume may be used. The approximate blood volume can be estimated by calculations based on body weight.

(*c*) *Study of material to be injected.* Assay the specific procoagulant activity of the material to be administered Express this activity in plasma equivalents, e.g , 1 ml. of the material is equivalent to 3 ml of normal plasma, unless well-defined standard units are available.

(*d*) *Administration of active material* This may be given by any of several parenteral routes [2] In the procedure outlined intravenous injection is used. If other than the intravenous route is used, appropriate modifications in the study of the recipient must be made

(*e*) *Studies of the recipient after administration·* Blood samples are obtained insofar as possible according to the following schedule (1) the first sample is collected within 5-10 minutes after completion of injection, (2) during the first 4 hours post-injection, samples are obtained at intervals of 30-60 minutes; (3) during the next 12 hours post-injection, samples are collected every 2 hours, (4) during the next 80 hours post-injection, samples

are collected at 12-hour intervals; (5) thereafter, samples are collected daily until the activity of the recipient's plasma has returned to the pre-injection level.

Blood volume determinations done during the post-infusion period are of value, and, if possible, should be made immediately after completion of the infusion. Subsequent determinations may be made periodically.

(f) *Analysis of samples:* Analysis for procoagulant activity may be done promptly. During the first 24 hours of study, there is a large number of samples; these may be stored at −20°C. before analysis for the procoagulant activity is carried out, provided that the procoagulant is stable.

Expression of Results and Interpretation: The results of the analyses are tabulated, and are also plotted on graph paper (abscissa shows time post-injection, ordinate shows the activity of the recipient's plasma in per cent of normal plasma). The expected maximal activity of the recipient's plasma post-injection is calculated from the assay and volume of the injected material and from the blood volume determinations

Further analysis of the data depends on the purpose of the study. The most rapid rate of loss from the plasma is usually immediately after maximal activity is reached. Beginning several hours post-injection, the loss is usually slower and at a more uniform rate The immediate rapid rate of loss has been considered as due mainly to equilibration of the procoagulant between the plasma and other body fluids, whereas the slower rate has been considered to reflect mainly metabolic utilization.

Precautions: Many factors may influence the rate of plasma clearance of administered procoagulants It is important to report all data collected. The status of the recipient prior to injection (pre-existing hemorrhage, trauma, etc.) may accelerate the clearance rate. Also, the source of the material injected may influence the clearance rate, e g , heterologous plasma or plasma products may show a different clearance rate from homologous products.

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2. Clinical Detection of Venous Thrombosis and Pulmonary Embolism

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Laboratory tests for the anticipation, detection and estimation of the extent of a thrombotic process are not as yet available. Diagnosis and prognosis depend solely on observation of clinical symptoms at the bedside. We shall deal here principally with venous thrombosis of the extremities and pulmonary embolism in obstetrics and surgery, covering the following points:

1. Basic concepts of the nature and development of thrombosis.
2. Date of appearance of the first clinical symptoms of superficial and deep thrombosis, and the significance of the two forms.
3 Symptomatology of deep venous thrombosis of the extremities.
4. Symptomatology of pulmonary embolism
5. Factors modifying or masking the thrombotic process.

1. *Basic Concepts of the Nature and Development of Thrombosis:* Clinical experience being the sole means of appreciating the severity of thrombosis, detailed observation of every patient's symptoms and signs is essential. Using as a criterion the duration of thrombotic symptoms, a rough estimate of the relative frequency of severe and benign thrombosis can be made, an important point in assessing therapeutic results. Table 1 shows the duration of thrombotic disease in 566 obstetrical and surgical

TABLE 1 *Duration of Development of Peripheral Vein Thrombosis Post-partum under Conservative Treatment with No Attempt to Directly Influence the Thrombotic Process*

Weeks	Thrombosis	
	Degree	Per cent occurrence in patients
1	mild	74 0
2	mild	1 2
3	severe	2 4
4	"	2 3
5	"	3 1
6	"	3 6
7	"	2 8
8	"	3 2
9	"	2 7
10	"	1 9
11	"	0 8
12	"	0 9
13	"	0 7
14	"	0 4

*patients** *whose treatment was purely conservative.* The course of the illness was simply observed while the patient was immobilized in bed and received no medication intended to influence the thrombotic process. The series covers the years 1943 to 1948 before anticoagulants were in use and corresponds to a thrombosis incidence of 2.8 per cent. During this period there were 14 cases of fatal pulmonary embolism. It is true that in many cases death occurred before the end of the first 3 weeks of thrombotic illness, but this does not affect the mean values for the duration of development.

In three-quarters of cases of thrombosis, 1 to 2 weeks of conservative therapy is all that is necessary. These patients recover spontaneously whatever is done for them. This is the group in which thrombosis can be avoided by early mobilization and controlled by coumarin derivatives. In one-quarter of cases, i e , 141 in the above series, thrombosis lasts 3 weeks or longer; it cannot be avoided by early mobilization, it cannot be controlled by coumarins but only by heparin therapy. Only on the basis of these cases of prolonged thrombosis, in which deep veins are invariably affected, and on the incidence of fatal pulmonary embolism can the value of a particular prophylaxis or therapy be assessed in the same clinic by the same observer. The duration and force of the thrombotic "impetus," or, in other words, the persistence in the organism of a tendency to intravascular coagulation, is thus established from the start for a given patient; it is already present when the first symptoms appear, although we have no means of estimating it accurately.

Our entire therapy remains purely symptomatic, for even anticoagulants, though able to increase blood fluidity temporarily, are unable to correct a proneness to accelerated coagulation Consequently, there are no therapeutic measures available that could shorten the period during which this tendency persists; therefore, the patient suffering from severe thrombosis remains in danger of relapse and is unable to return to work By maintaining a patient on anticoagulants, the tendency to coagulation is kept in check, but as soon as this medication is stopped the thrombus will re-form rapidly *without sticking firmly to the vein wall, therefore causing no* symptoms, and involving considerable risks for the patient. Only rest and a long period of careful prophylactic preparation before an operation can lessen the impetus of severe deep thrombosis, but this will not reduce the incidence.

Until more is known about the pathogenesis of the coagulation disorder, which is related to certain factors revealed only by the clinical history

* Observed at the University clinic of Obstetrics and Gynecology of Basle, Switzerland (Director Prof Dr Th Koller)

(trauma, infection, hemorrhage) and perhaps also to disturbance of enzymatic, endocrine and hepatic function, it will not be possible to develop more specific methods of therapy or to modify the prevailing status.

In order to compare results obtained in different clinics, the only valid criterion is the incidence of fatal pulmonary embolism confirmed at autopsy before and after the introduction of a particular prophylaxis or therapy for a similar type of operation or a similar group of patients. Patience and circumspection are essential before conclusions are drawn. A practical example follows:

Twenty thousand patients from one clinic were observed and treated by method X over 6 years and, distributed irregularly throughout this period, there were 141 cases of thrombosis including 14 deaths from embolism After instituting new methods of observation and therapy (method Y), 20,000 patients would have to be treated over a period of 6 years with only 4 deaths at the most before it could be asserted, with a margin of error of 5 per cent, that method Y is superior to method X (2 deaths at most for a margin of error of 2 per cent)

2. *Date of Appearance of First Clinical Symptoms of Superficial and Deep Thrombosis. Significance of the Two Forms·* Table 2 shows the date of appearance of the first symptoms of thrombosis post-partum or post-operatively. In a patient previously free of thrombosis, a superficial thrombosis appearing during the first few days after operation or delivery is generally benign, probably enhanced by disturbance of water and electrolyte metabolism If a superficial thrombosis appears only after 5-6 days, the prognosis is severe, since this almost invariably implies the presence of a concomitant deep thrombosis that has not yet given rise to clinical symptoms. Thus, surgical or obstetrical patient in whom a superficial thrombosis is noted on the 8th day without signs of deep venous thrombosis, may nevertheless be in danger of imminent death from pulmonary embolism, from an asymptomatic floating thrombus in a deep vein

Prolonged pre-operative preparation (8-14 days) may delay the appearance of thrombosis *Once this is realized and treatment undertaken* accordingly, heparin may be used with diminished risk of hemorrhage

3. [illegible] he symptoms of [illegible] thrombosis, in [illegible] utopsy, we may establish the following points.

(a) Deep thrombosis, not influenced by medication, nearly always *gives rise to symptoms; fatal pulmonary embolism without warning signs* of deep thrombosis is very rare

(b) Symptoms are manifold and often non-specific, they must be interpreted in relation to the particular clinical condition of the patient

TABLE 2 *Per cent Occurrence of First Signs of Thrombosis or Embolism Post-operatively or Post-partum*

Day	Post-partum: Per cent of patients with signs of superficial thrombosis	Post-partum: Per cent of patients with signs of deep thrombosis	Post-operatively: Per cent of patients with signs of superficial thrombosis	Post-operatively: Per cent of patients with signs of deep thrombosis
1	21	—	15	—
2	19	2	5	1
3	17	4	5	1
4	13	4	5	6
5	11	4	25	7
6	8	8	20	7
7*	6	16	5	9
8	4	10	4	10
9†	—	9	3	8
10	—	9	3	8
11	—	9	3	16
12	—	7	3	10
13	—	6	3	10
14	—	5	3	5
14	—	4	3	2

* *Post-operatively*, the highest incidence of fatal embolism was noted on this day.
† *Post-partum*, the highest incidence of fatal embolism was noted on this day

(c) The chief symptoms observed may be subdivided into general, pain and functional symptoms, and symptoms of stasis. These vary widely according to the site of the thrombosis. Usually only a few are present

(1) *General symptoms*· agitation, malaise, chills, weakness, prostration, ill feeling, elevated pulse, plateau pulse, fever. Embolism, of course, confirms the presence of thrombosis. Thrombosis in the left leg veins is 2-3 times more common than in the right

(2) *Pain symptoms* (reaction of the venous wall, generally lasting 5-15 days) Spontaneous pain or pain on palpation of a deep vein, pain in the region of the calf or plantar arch (spontaneous, on palpation or on flexion of the foot); pain on inflation of the cuff of a blood pressure apparatus to 150 mm Hg; vague sensations of burning, cramps, paresthesia and heaviness in the entire leg, in the calf, in the region of the uterus or rectum, or in the region of the bladder, sometimes accompanied by retention of urine.

(3) *Symptoms of stasis* (appearing after venous obstruction often only during the course of the 2nd week) Fluid retention; infiltration of the parametrium and rectum; edema of the flank, vulva, inguinal fold, leg, thigh, calf, ankle and foot; pitting of the calf and thigh; hydrarthrosis of the knees, glossy skin over the tibia, cyanosis and marble discoloration of the skin of legs, fingers and feet, swelling of superficial veins in the thigh.

(4) *Functional symptoms:* Meteorism, ileus, subileus; constipation; peritoneal irritation, urinary retention; sometimes local elevation of skin temperature; disappearance of the pilo-erector reflex in response to the cold test; autonomic nervous disturbances (moist skin, etc.); increased venous pressure; delayed absorption of an intra-epidermal blister.

The suspected venous pathways should be gently palpated daily or even several times a day, because a severe deep thrombosis may form within a few hours. Furthermore, careful observation of fluid balance gives a good guide to the course of the thrombotic disease and enables the dates of crisis and lysis, reflecting the tendency to coagulation, to be established.

Under conservative therapy, crisis and lysis, due to thrombotic flare-up, may occur several times in succession during the course of the illness, and an isolated occurrence is rare. Under well-conducted treatment with heparin, the only medicament capable of checking the severe thrombotic impetus, there is only one crisis and lysis forcibly induced These phenomena are observed most clearly in post-partum thrombosis.

4. *Symptomatology of Pulmonary Embolism:* The greater the clinical experience of the physician and the better he is able to observe and supervise his patient, the lower the mortality. Pulmonary embolism is very rarely fatal at the first attack but is usually preceded by symptoms of thrombosis or minor episodes of embolism. This is the lesson to be drawn from study of the 300 fatal cases of pumonary embolism quoted in section 3 The symptoms, subjective and often atypical, must be interpreted in regard to the clinical situation of the patient after surgery or childbirth. Against this background, a diagnosis of embolism must be made on appearance of any, more or less sudden, and inexplicable change in the general state malaise, chills, tachycardia, fear, prostration, agitation, pricking sensations in the cardiac region, cardiac oppression, cough, pallor, attacks of vertigo and fainting, fever, stabbing pains in the thorax and retrosternal region, etc.

Numerous forms of pulmonary embolism with atypical symptoms may develop during the course of deep thrombosis and the physician should not wait for the patient to cough blood or develop painful dyspnea before making the diagnosis. A patient habitually in good health is not suffering from tuberculous pleurisy if he experiences stabbing pains in the thorax and dyspnea 10 days after an operation such as herniotomy He is suffering from embolism and it is unwise to endanger his life still further by mobilizing him for auscultation or chest x-ray examinations without first starting him on heparin therapy.

5. *Factors Modifying or Masking the Thrombotic Process* Symptoms may be attenuated by pre-operative preparation lasting 6-10 days or longer, this is observed even in patients in apparently good general health The preparation consists primarily in physical and mental rest, the patient being

most of the time in bed but allowed up for short walks at frequent intervals, in gentle massage of the legs, very light breathing exercises, a light fat-free diet, and habituation to hospital surroundings. Such measures do not reduce the incidence of deep thrombosis but, as mentioned above, they do diminish the severity and, above all, the risk of fatal embolism. They also have the effect of delaying the appearance of thrombosis until a later date after the operation.

Symptoms may also be masked, partially or entirely, in patients who have received anticoagulants or fibrinolytic agents in inadequate dosage or in adequate dosage for too short a period, or who have been given anti-inflammatory medication. The thrombus develops but does not adhere to the venous wall in patients undergoing medication when the dosage of anticoagulants is too low, and in patients treated with anti-inflammatory agents, or after too short periods of medication even though in adequate dosage; the thrombus floats and becomes elongated and hence causes no symptoms until embolism has occurred. Accordingly, once the patient has been started on one of the above treatments—anticoagulants for instance—it is no longer possible to rely on observation of clinical symptoms, since these are masked or no longer manifest.

In order to avoid death during anticoagulant therapy from pulmonary embolism caused by an asymptomatic floating thrombus, the following two rules, established empirically, should be observed:

1. Anticoagulant therapy should be continued for at least 3 weeks, the patient should not return to work and should avoid getting tired at the end of this therapeutic phase to prevent a recurrence.

2 Insufficient dosage (to prevent hemorrhage but permitting the formation of a silent floating thrombus) should be avoided at all costs. We have observed eight cases of fatal embolism resulting from failure to observe these rules; all showed, before sudden death, a great improvement of their general condition and diminishing or absent signs of local thrombosis masked by inadequate therapy.

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3. Clinical Detection of Thromboembolic Disease

H. L. ISRAEL

Evidence of the inadequacy of clinical diagnosis of thromboembolism is afforded by a recent study at Philadelphia General Hospital. Pulmonary emboli were found on necropsy in 18 per cent of deaths on the medical service, less than 25 per cent of these had been recognized and treated antemortem

Yet most fatal embolism is preceded by warning signs, either in the form of venous thrombosis or minor pulmonary emboli. Unfortunately, these manifestations are often mild and are frequently overlooked by physicians. The accuracy of diagnosis of thromboembolism can be improved by constant attention to the possibility of venous thrombosis, especially in the lower extremities, and by constant alertness to symptoms indicative of pulmonary embolism. The diagnosis is obviously more firmly based when both are present, but commonly the diagnosis must be based on incomplete evidence. Characteristic symptoms, radiologic and electrocardiographic changes often permit a diagnosis of pulmonary embolism in the absence of signs of phlebitis, but repeated careful examinations of the lower extremities will often disclose signs when cursory examination is regarded as negative

Recognition of Deep Vein Thrombosis

Venous thrombosis is especially frequent in patients immobilized by congestive heart failure, strokes, surgical operations and severe injuries, but it is also common and should be sought for especially after minor injuries to the extremities, following use of intravenous catheters, and in patients with neoplasms, anemias, and other debilitating illnesses

Pulmonary emboli are believed to originate in the veins of the lower extremities in approximately 90 per cent of cases In one recent pathologic study[1] of pulmonary embolism on a surgical service, thrombi were demonstrable in thigh or leg veins in every case. The calf and iliofemoral veins were most often involved Thrombosis was unilateral in 23 per cent and bilateral in 77 per cent

Clinical evidence of venous thrombosis is much less often demonstrable. Signs of lower limb thrombophlebitis were found in 70 per cent of cases of pulmonary embolism in a Philadelphia study[2] (table 1) and somewhat less frequently in English studies [1,3] Clinical evidence of phlebitis may appear a day or two after embolism, if a diagnosis of pulmonary

TABLE 1. *Source of Pulmonary Emboli, Graduate Hospital, 1955-1957*

Clinical evidence of lower extremity phlebitis	58 cases
Clinical evidence of upper extremity phlebitis	2 "
Suspected pelvic phlebitis	4 "
Suspected cardiac origin	7 "
Source unknown	13 "

embolism is made early and anticoagulant treatment instituted, the signs of thrombophlebitis may be averted

Other sources of embolism include the pelvic and other abdominal veins, the tricuspid and pulmonic valves and the veins of the upper extremities; and careful consideration should be given these sites when changes in the lower extremities are lacking.

Venous thrombosis may produce local symptoms, constitutional symptoms or they may be asymptomatic. The principal local manifestations of lower limb venous thrombosis are pain in the foot or leg on walking, sensations of tightness in the calf, and edema. The constitutional symptoms include fever, tachycardia and apprehension Fever is usually of low grade, but may occasionally reach 101° or 102°F. Tachycardia disproportionate to temperature elevation is a characteristic feature; and venous thrombosis should be suspected in the post-operative patient who exhibits unexplained tachycardia. Apprehensiveness without obvious basis is often noted in patients prior to thromboembolic manifestations, but a study of this phenomenon in post-operative patients indicated that this symptom was inconstant and unreliable as an indication of venous thrombosis or pulmonary embolism.

In many instances symptoms are absent, and reliance must be placed on careful physical examination Numerous maneuvers have been devised to aid in diagnosis Comparison of the circumference of the legs is valuable, taking care that the tape measure is applied at corresponding points of the two legs. As a rule, however, edema is a late sign of venous thrombosis and *tenderness can usually be demonstrated earlier This may be elicited* by point pressure along the course of the femoral veins, in the popliteal spaces, over the Achilles tendons, and by palpation of the feet

Demonstration of pain by inflation of a sphygmomanometer cuff to a pressure of 150 mm. has been useful in the experience of some, but we have found this procedure unreliable A familiar and valuable test is the maneuver described by Homan Forceful dorsiflexion of the foot will frequently elicit pain in the calf if deep phlebitis is present. Unfortunately in many cases of phlebitis this sign is not present, but when demonstrable it is highly reliable. The most sensitive indication of deep venous thrombosis appears to be the maneuver described by Moses [4] This consists of

firm calf compression in the antero-posterior direction with the finger tips. If this demonstrates persistent localized muscle tenderness, two additional steps are necessary to exclude myositis and peripheral neuritis. A brief neurologic examination is made, and the calf is compressed in the lateral direction, which is equally painful in the presence of myositis and neuritis. In venous thrombosis, lateral compression is relatively painless compared to postero-anterior compression.

Other valuable signs of deep vein thrombosis are distended pretibial and pedal veins.

Varicose veins and superficial thrombophlebitis are not regarded as signs of deep phlebitis but are present in approximately a third of patients with pulmonary embolism. Superficial vein disease predisposes to deep phlebitis, and its presence should lead to especially careful watch for evidence of deep vein thrombosis

Venograms, obtained by injections of radio-opaque dyes into the veins of the foot, have proven an aid to diagnosis in expert hands. Anatomic variations in the venous patterns are so common, however, and filling of all channels so difficult, that few radiologists have achieved proficiency in demonstration of phlebitis by this technic. False negative and false positive reports are both frequent with this method of study

The application of isotope technic to demonstrate venous obstruction is under investigation, but reliable and practical methods have not yet been developed

Recognition of Pulmonary Embolism

Clinical examination: Failure to recognize pulmonary embolism is often due to lack of familiarity with the various clinical manifestations it may produce The symptoms are predominantly respiratory in 45 per cent of cases, cardiovascular in 40 per cent, and abdominal or neurologic in the remainder The respiratory diseases simulated include pneumonia, atelectasis, pleurisy, neoplasm and tuberculosis The cardiovascular disease most often confused is acute myocardial infarction, but recurrent pulmonary emboli may simulate angina or may insidiously produce cor pulmonale. Pulmonary infarcts on the diaphragmatic surfaces may cause severe abdominal pain, resembling that of acute surgical conditions, especially acute cholecystitis Pulmonary embolism in elderly patients not infrequently causes transient cerebral insufficiency with symptoms of syncope or transient paresis

The principal symptoms of pumonary embolism are dyspnea and pleuritic pain, acute and episodic in character Hemoptysis, anginal pain and hypotension are less frequent but important manifestations.

The clinical signs of a massive embolism producing acute cor pulmonale include shock, cyanosis, sweating. The cervical veins may be distended and pulsating and there may be increased pulsation in 2nd and 3rd interspaces to the left of the sternum, where a loud systolic murmur and a gallop may be audible. In most cases, physical examination of the heart and lungs is less rewarding than examination of the extremities. Pleural friction rubs and local chest tenderness are characteristic signs which may be found in a quarter of patients.

Thoracentesis is occasionally a valuable aid to diagnosis. About 50 per cent of effusions due to embolism are blood-tinged and demonstration of sanguineous fluid may provide strong support for an embolic rather than infectious etiology of pleural effusions.

Electrocardiographic examination: The first description by McGinn and White in 1935, of a characteristic electrocardiographic pattern following pulmonary embolism called attention to changes reflecting acute pulmonary hypertension manifested by the development of an S wave in lead I and a Q wave and inverted T wave in lead 3 These classical changes of acute cor pulmonale, however, occur in only 5 per cent of cases of embolism. Studies in recent years have disclosed that a variety of patterns occur following pulmonary embolism. When serial tracings permit detection of minor and transient changes, it is possible to recognize alterations related to the embolism in 70 per cent of cases [2,5] Patterns of positional change resulting from acute pulmonary hypertension are commonly observed in patients with previously normal electrocardiograms These changes include clockwise rotation manifested by S waves over the left precordium, R waves in AVR, S waves in lead I and Q waves in leads III and AVF

In older patients with previous myocardial damage, embolism more commonly results in electrocardiographic changes reflecting the decreased cardiac output and diminished coronary flow, producing changes easily confused with those of myocardial infarction These include ST segment depressions and T wave inversions over the left precordium as well as T wave inversions in the right precordial leads Other characteristic abnormalities associated with pulmonary embolism are transient right bundle branch block, and atrial fibrillation

Electrocardiographic changes are almost invariably present in patients exhibiting syncope, hypotension or congestive failure, but are commonly present even when these circulatory manifestations are absent. Although the electrocardiographic changes appear early and usually quickly disappear, a remarkable and unexplained feature is the persistency of the changes in some cases for many weeks

Radiologic examination· Radiographic abnormalities occur in over 80 per cent of cases,[6] occasionally not becoming manifest until 1 or 2 days after

embolism. The changes may be pleural, diaphragmatic, parenchymal cardiovascular.

(1) Pleural effusion, as a rule small or moderate in size, occurs almost one-half of cases. Occasionally, massive pleural effusions occur

(2) Elevation of the diaphragm is demonstrable in a third of ca often representing splinting due to painful respiration.

(3) Parenchymal densities are due more to blood extravasation, br chial obstruction and atelectasis than to extensive necrosis. Differentiat from pneumonias and tumors may be impossible at the initial examinati but serial studies will often demonstrate the characteristic pattern follow embolism of healing by formation of linear scars Only about 10 per c of infarct shadows have the classical triangular configuration

(4) Rarely, following occlusion of a main pulmonary artery, there n be increased translucency of the entire lung due to oligemia. More oft dilatation of the pulmonary artery trunk or secondary hilar vessels may demonstrable as a result of pulmonary hypertension. Right ventricu dilatation resulting from chronic pulmonary hypertension may be noted patients with recurrent pulmonary embolism. Venous angiocardiogra has been reported useful in confirming diagnoses of pulmonary emboli However, the applicability of this method appears to be limited.

Laboratory tests. The clinical laboratory provides relatively little ass ance in the diagnosis of thromboembolism. A helpful test in differentiat of pulmonary and myocardial infarction is the determination of ser (SGOT) transaminase, since the level of this enzyme does not rise follc ing pulmonary embolism Wacker and Snodgrass[7] have pointed out t the triad of lactic dehydrogenase elevation, increased serum bilirubin a normal SGOT transaminase is characteristic of pulmonary embolism our experience, enzyme studies have proven only occasionally helpful the diagnosis of pulmonary embolism

Recently, respiratory function studies have been utilized in the diagnc of pulmonary embolism. Robin and his associates[8] noted marked arte oxygen unsaturation after embolism This may prove useful in differen tion from acute myocardial infarction which causes only slight hypoxen These investigations have also described marked arterial-alveolar CO_2 t sion differences due to continued ventilation of alveoli unperfused beca of the embolism. When this difference is marked, occlusion of a m pulmonary artery is suspected.

Conclusions

Fatal pulmonary embolism is preceded in a majority of instances warning signs—either evidence of venous thrombosis demonstrable careful examination of the lower extremities, or by minor episodes

pulmonary embolism often recognizable by informed electrocardiographic and radiographic interpretation. Although full ultilization of these methods will improve diagnostic accuracy, clinical diagnosis of thromboembolism will remain unsatisfactory until new approaches are developed. Fruitful fields of investigation appear to be those of radioisotope tests for venous obstruction, and more specific enzyme and respiratory function tests for pulmonary embolism.

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Appendix

1. Synonyms for Components Influencing Blood Coagulation

A. *Blood Clotting Factors*[1]

Factor I	Fibrinogen
Factor II	Prothrombin
Factor III	Thromboplastin (tissue)
Factor IV	Calcium
Factor V (VI)	Proaccelerin (Accelerin) Accelerator Globulin (AcG)
Factor VII	Proconvertin Serum Prothrombin Conversion Accelerator (SPCA) Stable Factor Autoprothrombin I
Factor VIII	Antihemophilic Factor (AHF) Antihemophilic Globulin (AHG) Platelet CoFactor I Facteur Antihémophilique A
Factor IX	Plasma Thromboplastin Component (PTC) Christmas Factor Platelet CoFactor II Autoprothrombin II Facteur Antihémophilique B
Factor X	Stuart Factor Prower Factor Autoprothrombin C[2]
Factor XI	Plasma Thromboplastin Antecedent (PTA)
Factor XII	Hageman Factor
Factor XIII[3]	Fibrin Stabilizing Factor (FSF) Laki-Lorand Factor (LL. Factor) Fibrinase

B. *Blood Clotting Activities*[4]

Prephase accelerator (PPA)
Activation Product (Contact Factor)
Thrombin E (Esterase Thrombin)
Autoprothrombin II A (Anticoagulant)
Factor R (platelet adhesive and clumping factor)
Vascular Factor (V.F.)
Thrombocyte Agglutinating Factor (TAg)
Thorium Vulnerable Factor (TVF)
Labile Serum Factor
Vasculokinase (VK)
Serum Thrombotic Accelerator (STA)
Thromboplastin Generation Accelerator (TGA)

C. *Blood Cell Derived or Adsorbed Factors*[5,6,7]

Erythrocytes
Erythrocytin
Platelets
Platelet Factor
1. *(Factor V)*
2. (Fibrinoplastic)
3. (Thromboplastic)
4. (Antiheparin)

Platelet Co-thromboplastin (Factor VII)
Fibrin Stabilizing Factor
Platelet Antifibrinolysin
Prothrombin
Factor VIII
Fibrinogen
Thrombosthenin

D *Inhibitors*[7]

Antithrombins
I (Fibrin)
II (Heparin Cofactor)
III (Progressive)
IV (Activity formed during thrombin formation)
V (Antifibrinogen)
VI (Fibrinogenolytic Products)
Antithromboplastin
Contact Factor Inhibitor

Antiproconvertin
Antihemophilic Globulin Inhibitor

E. *Fibrinolytic Factors*[8,9]
Profibrinolysin-Fibrinolysin
Plasminogen-Plasmin
Proactivator-Activator
Tissue Activator
Urokinase
Streptokinase

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